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The Relationships between Diet, Physical Activity and Dual Plane (3D) Dual Energy X-Ray Absorptiometry (DXA) Bone Outcomes in Pre-pubertal Girls

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ABSTRACT

Osteoporotic fractures are a leading cause of morbidity in the U.S. Bone mass levels during childhood play a key role in determining peak bone mass and hence the risk of osteoporosis in later life. The aim of the study was to evaluate how key bone nutrients and organized physical activity affect bone outcomes in pre-pubertal girls using Dual Plane Dual X-Ray Absorptiometry (DXA). This cross-sectional analysis used a subset of participants (n=50) from a longitudinal study of bone growth in relation to physical activity. Dietary data were collected using the Youth/Adolescent Questionnaire (YAQ, 1995) and organized activity was recorded semi-annually to yield annual means (hours per week). Paired postero-anterior (PA) and supine lateral lumbar spine (LAT) Dual X-Ray Absorptiometry (DXA) scans provided L3 PA bone mineral density (PABMD), PA vertebrae width (PAWIDTH), bone mineral content (PABMC, LATBMC), LAT vertebral height (LATHEIGHT), LAT vertebral depth (LATDEPTH), paired vertebral volume (PALATV) and bone mineral apparent density (PALATBMAD). Bone strength in axial compression (PALATIBS) and fracture risk index (FRI) were calculated. Multiple linear regressions were used to analyze the association between key bone nutrients, level of physical activity, and bone outcomes. Carbohydrate, fiber, and zinc intake significantly correlated with bone outcomes. After accounting for age, height, and activity, focal nutrients were not significant factors for prediction bone outcomes. Physical activity was positively associated with PABMD, PABMC, PAWIDTH, LATBMC, PALATIBS after adjusting for age, height, and all the key nutrients. We found physical activity has greater explanatory value than nutrient intakes for bone content, density, geometry and strength in well-nourished pre-pubertal girls.

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Table of Contents

Abstract

Title Page

Copy Right

Acknowledgements

List of Illustrative Material

1. Literature Review

Osteoporosis is one of the leading causes of morbidity for women (Lippuner, Johansson, Kanis, & Rizzoli, 2009). Low bone mineral acquisition during childhood and adolescence increases the risk of osteoporosis later in life. Peak bone mass is negatively associated with the risk of osteoporosis, and a large percentage of peak bone mass is accumulated during childhood and adolescence. Hence, bone mass accretion during childhood and adolescence plays a key role in influencing the risk of osteoporosis in later life (Huncharek, Muscat, & Kupelnick, 2008). In addition to genetic factors, modifiable factors, including diet and physical activity play critical roles in bone growth and maintenance in children and adolescents (Rizzoli, Bianchi, Garabedian, McKay & Moreno, 2010). It is important to have an adequate diet and adequate physical activity for bone health, particularly during adolescence, because bone is more responsive to nutrients and mechanical stimuli for bone development during this period compared to adulthood (Bonjour, 2011). To our knowledge, no study has specifically investigated diet and physical activity associations with indices of lumbar spine bone mass, geometry and strength using paired PA/lateral lumbar spine Dual-energy X-ray absorptiometry (DXA) scans in pre-pubertal girls. The aims of this study are: first, investigate the associations between 3rd lumbar vertebrae (L3) mass, geometry and strength and dietary intake of energy, macronutrients, key bone micronutrients including calcium, vitamin D, vitamin C, vitamin K, vitamin A, B vitamins, phosphorus, magnesium, iron, and zinc; second, evaluate the association between L3 and physical activity.

1.1 Bone

1.1.1 Importance of Bone Health

Osteoporosis is a disease characterized by low areal bone mineral density (aBMD). World Health Organization (WHO) criteria defined osteoporosis as aBMD that falls 2.5 standard deviations below the average aBMD of healthy young women (World Health Organization, 2007). Osteopenia is a condition where an individual's bone mineral density is below normal, but has not reached osteoporosis. BMD test results are expressed as T-scores and Z-scores. T-scores represent the number of standard deviations (SD) of patient's test results compared to reference standards for female, Caucasians, 20-29 years old contained in the National Health and Nutrition Examination Survey (NHANES) III database. Z-scores are comparisons of SDs against gender and race specific, age adjusted databases. Z-scores should be used for diagnoses in children and in males less than 50 years of age (The International Society for Clinical Densitometry, 2005)

Osteoporosis and osteoporotic fractures are leading causes of morbidity in industrialized countries. About 20% of men and 50% of women will suffer from osteoporotic fracture after the age of 50 (Lippuner, Johansson, Kanis, & Rizzoli, 2009). The most common fracture sites are spine, hip, forearm and proximal humerus (World Health Organization, 2007; Looker, Melton, Harris, Borrud, & Shepherd, 2010). Data from the National Health and Nutrition Examination Survey (NHANES) 2005-2006 showed that an estimated 5.3 million older men and women met the WHO definition of osteoporosis at the femoral neck, and 34.5 million had osteopenia.
Age is a strong factor that has been negatively related to aBMD, particularly in women. For women, mean aBMD is significantly lower in each successive age decade (Looker, Melton,

Harris, Borrud, & Shepherd, 2010). Osteoporotic fracture can lead to financial, physical and psychosocial consequences (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, 2001). The consequences of osteoporosis make the prevention of the disease essential to maintain health and quality of life (Looker, Melton, Harris, Borrud, & Shepherd, 2010).

Peak bone mass is negatively associated with the risk of osteoporosis, and a large percentage of peak bone mass is accumulated during childhood and adolescence. Bone mass levels during childhood and adolescence play a key role in determining peak bone mass and hence the risk of osteoporosis in later life (Huncharek, Muscat, & Kupelnick, 2008). Bone mineral accretion during childhood and adolescence not only protect against bone disease later in life, but also prevent bone fracture during childhood and adolescence (Bianchi, 2007).

1.1.2 Bone Composition and Homeostasis

Bone is a highly specialized and dynamic living organ. It is composed of mineralized/inorganic and unmineralized/organic connective tissue, highly specialized cells, and cavities and spaces (Manolagas, 2000).

1.1.2.1 Bone Composition---Biochemical component and type of cells.

The inorganic component ($(Ca, Na, Mg)_{10}(PO_4,HPO_4,CO_3)_6(OH,Cl,F)_2$) of bone is primarily composed of minerals including calcium, phosphate, carbonate, magnesium, and sodium, which

provide strength and stiffness to the human body (Kay, Young & Posner, 1964; Rey, Renugoplakrishan & Collins, 1991; Ellliott, 1994). The organic component of the skeleton, called osteoid, is composed of matrix proteins, which are mainly type I collagen and minor amounts of other proteins (Bouxsein, 2005; LeGeros, 2008). The organic component of bone provides elasticity.

On the cellular level, bone is formed by four types of cells: osteoblasts, osteocytes, bone-lining cells, and osteoclasts. Derived from pluripotent mesenchymal stem cells (MSCs) in bone marrow, osteoblasts are the chief bone forming cells (Levine, 2012). Osteoblast differentiation and function are tightly regulated by many factors. Runt-related 2 (Runx2), a transcription factor, is thought to be the master regulating factor of osteoblasts. The Level of Runx2 expression determines the number of osteochondral progenitor cells that develop into osteoblasts. Runx2 also regulates multiple genes which determine osteoblast phenotype (Karsenty, 2008). In addition, rux 2 controls the level of osteocalcin, an osteoblast-specific hormone that is secreted only by mature osteoblasts that regulates energy metabolism (Lee, et al., 2007). Activating transcription factor-4 (ATF4), another transcription factor, is critical for osteoblasts to become fully functional (Karsenty, 2008). Osteoblasts stimulate the production of type I collagen and other bone matrix proteins (Karsenty, 2008).

Osteoblasts play a crucial role in bone formation. Bone formation includes the following three phases: osteoid matrix production, maturation, and mineralization (Hadjidakis & Androulakis, 2006). Osteoblasts form a non-mineralized extracellular matrix (osteoid), to cover the

mineralized extracellular matrix of bone tissue. Osteoid is mainly composed of the following type I collagen, osteocalcin, and osteonectin. In addition to matrix formation, osteoblasts are also important for bone mineralization through the process of hydroxyapatite deposition. Osteoblasts express high amounts of alkaline phosphatase, which plays an important role in bone mineralization (Ducy et al., 1996). The mineralization process results in the lightweight but hard skeletal tissue (Hall, 2005). Osteoid matrix determines the volume and shape of bone while matrix mineralization alters bone density (Manolagas, 2000).

Osteoblasts stay active in human bone for approximately three months (Manolagas, 2000), and then a small proportion of them are incorporated in the lacunae of mineralized bone matrix as osteocytes, which have an estimated half-life of 25 years (Frost, 1963). About 95% of mammalian bone is composed of osteocytes (Frost, 1960). Osteocytes function as force and stress sensors and in signal conduction (Franz-Odendaal, Hall & Eckhard Witten, 2005). They also sense changes in levels of hormones (estrogen and glucocorticoids) in interstitial fluid flow through canaliculi (Weinstein, Jilka, Parfitt & Manolagas, 1998). Thus, osteocytes are crucial for bone structure and integrity maintenance (Franz-Odendaal, Hall & Eckhard Witten, 2005). Like osteocytes, bone lining cells are derived from osteoblasts. The surface of bone is covered by a layer of unmineralized collagen matrix. Bone lining cells lay on top of the collagen matrix layer (Parfitt et al., 1996).

Osteoclasts are derived from hematopoietic stem cells. Induced by macrophage colony-stimulating factor (M-CSF), hematopoietic stem cells proliferate and differentiate into

macrophage colony-forming units (CFU-M). By binding receptor activator of NF-κB ligand (RANKL) to its receptor RANK, which is expressed on CFU-M, the osteoclast precursors continue to differentiate into multinucleated osteoclasts. They eventually become activated and develop into mature large osteoclasts (Chambers, 2010; Lerner, 2006).

Osteoclasts function as mineralized bone matrix resorption cells (Chambers, 2010) with an average lifespan of two weeks (Manolagas, 2000). Bone resorption is a process of bone degrading. To degrade bone tissue, osteoclasts create an isolated microenvironment between themselves and the skeletal surface. The cells then secrete HCl to acidify and demineralize the organic matrix of the bone. The process of degradation is facilitated by lysosomal enzyme cathepsin K. Net bone resorption is controlled by the number of osteoclasts and by the matrix-degrading capacity of individual osteoclasts (Zou & Teitelbaum, 2010). The functions of osteoclasts are matched by their morphological features: large multinucleated cells with finger-shaped projections of the membrane and abundant mitochondria, lysosomes, and ribosomes (Roodman, 1996)

1.1.2.2 Bone Homeostasis--Bone Modeling and Remodeling

Bone modeling, also referred to as bone construction, continues from birth until early adulthood. During the development and growth period, bone modeling changes the size, shape and density of bone by osteoblasts depositing new bone tissue without previous bone resorption (Seeman & Delmas, 2006; Manolagas, 2000). During bone modeling, bone formation and resorption is not balanced, greater relative formation leads to increasing bone size and density.

Bone remodeling is the process of bone reconstruction, which occurs continuously throughout the lifetime of vertebrates' skeletal tissue. This process renews approximately 10% of bone tissue each year (Lerner, 2006). Different from bone modeling, new skeletal tissue forms through the action of osteoclasts' bone resorption followed by osteoblasts' bone formation. The purpose of bone remodeling is to replace the older or damaged bone with newly formed bone to repair skeletal damage, regulate skeletal shape, size, mass, and quality as well as to perform the skeletal function of maintaining blood mineral and acid/base homeostasis (Parfitt, 2002; Lerner, 2006). While bone modeling leads to an excess in bone formation compared to bone resorption, the two processes are balanced during bone remodeling. Age related hormonal changes, metabolic bone disease, or use of medication that interferes with bone accrual can cause negative balance in bone formation and resorption, which further leads to increased risk of osteoporosis and fracture.

Bone remodeling is initiated by osteoblast-dependent osteoclast activation. The activation processes are carried out in the "basic multicellular units" (BMU) (as observed via tetracycline-based histological analysis of bone remodeling). Bone remodeling begins when the fully activated osteoclasts attach to bone. They form resorption lacunae and leave a mononuclear cell to clean up the organic matrix. When resorption is accomplished, osteoblast precursors are brought to the lacunae they develop into fully active osteoblasts and form new bone in the lacunae under the regulation of insulin growth factor-1 (IGF-1) and transforming growth factor-β (TGF- β) (Lerner, 2006). The mature mononuclear osteoblasts attach to deposit osteoid and mineralize it, which forms newly synthesized matrix. Some osteoblasts differentiate into osteocytes, while the rest transform into bone lining cells. This process is considered a reversal period, and is followed by a resting phase (Glustina, Mazziotti & Canalis, 2008; Lerner, 2006).

The processes of bone modeling and remodeling modify the external/internal shape, size and structure of bone by depositing new tissue and removing old tissue from the bone surface (endocortical, intracortical, trabecular). They lead to the thickening of bone during growth and thinning during aging (Seeman & Delmas, 2006).

1.1.3 Type of Bone and Functions

Adult human skeletal tissue is composed of 80% cortical bone and 20% trabecular bone. Though they share the same chemical composition, the two bone types have different structures at macroscopic and microscopic levels (Ammann & Rizzoli, 2003; Hadjidakis & Androulakis, 2006). Cortical bone, also called compact bone, is dense, solid, and lines the outer layer of all bone structures. It has a relatively slow turnover rate and a high resistance to bending and torsion. Long bones are mainly composed of cortical bone, and they function as levers to provide support, protection and locomotion to the body. They also contribute to metabolic responses, particularly when there is severe or prolonged mineral deficit (Ammann & Rizzoli, 2003; Hadjidakis & Androulakis, 2006). Trabecular bone, which is also called cancellous or spongy bone, is less dense, more flexible, and has a higher turnover rate than cortical bone. The higher surface area to mass ratio makes it more suitable for mineral exchange (Ammann & Rizzoli, 2003). Trabecular bone plays an important role in metabolic response and mineral supplies, thus there is a higher risk of developing osteoporosis under mineral deficiency states. It also participates in support and locomotion, particularly in energy absorption, with vertebrae as an

example. The porous structure of vertebrae allows them to function like springs to absorb energy by deforming (Seeman & Delmas, 2006; Hadjidakis & Androulakis, 2006).

Due to the greater surface area to volume ratio, trabecular bones have a higher remodeling and turnover rate than cortical bones. With increasing age, remodeling continues and trabecular bones perforate or even disappear. Remodeling on the endocortical surface becomes more active and eventually causes the "trabecularization" of the cortical bone. The cortical porosity further increases bone loss and risk of fracture (Seeman & Delmas, 2006). Different bones have different cortical to trabecular tissue ratios. This ratio is 1:3 in vertebrae, 1:1 in the femoral head and 19:1 in the radial diaphysis (Ammann & Rizzoli, 2003).

1.1.4 Bone Strength, Fracture, and Determinant Factors

Bone strength is determined by its content and structure (Currey, 2002). The organic component, such as type I collagen, provides bone its flexibility, so bones are able to absorb impact by deforming. The inorganic component, crystals of calcium hydroxyapatite, provides stiffness, so bone can support loading and locomotion. In addition to these characteristics, the optimal structure of skeletal tissue at both microscopic and macroscopic levels makes it possible for bones to be strong and light to facilitate movement and support loading (Saito & Marumo, 2010).

Bones are likely to fracture when the imposing mechanical loads are similar to or greater than their strength (Duan, Seeman & Turner, 2001). Osteoporosis increases the risk of bone fracture. Approximately 40% of patients with osteoporosis will experience fracture during their lifetime.

Spine, hip and wrist are the sites where fragility fractures most commonly occur (Rachner, Khosla & Hofbauer, 2011).

An epidemiological study showed that the incidence of fracture reached peaks at both puberty and old age. Children and adolescents with low aBMD and peak bone mass are thought to be at a higher risk of osteoporosis, which is associated with increased risk of fracture (Palacious 2006; Bianchi, 2007). The Dunedin Multidisciplinary Health and Development Study, a longitudinal study which followed 601 participants born in Dunedin, New Zealand from birth to 18 years, found that half of the participants had at least one fracture by age of 18, and many of them reported repeated fractures. The peak time when girls reported experiencing at least one fracture was between 9-14 years old (and 13-14 years for boys). In this cohort, the wrist/forearm were the most common fracture sites, which accounted for more than one quarter of all the fractures (Jones, Williams, Dow & Goulding, 2002)

1.1.5 Puberty and Bone Development

Puberty plays a critical role in bone development and bone mass accumulation (Yilmaz et al., 2005). Bone mass is progressively built up during childhood and adolescence. Approximately 90% of peak bone mass is accumulated during this period of life, which determines the risk of osteoporosis occurrence in later life (Bianchi, 2007). Pre-pubertal children show similar aBMD values at the lumbar spine in both sexes. Nevertheless, aBMD is elevated sooner in girls than in boys due to the earlier onset of the pubertal growth spurt in girls (Saggese, Baroncelli & Bertellponi, 2002).

1.1.5.1 Hormonal Changes during Puberty that Affect Bone Development

The processes of bone modeling and remodeling are intricate and tightly controlled by multiple hormones (Chambers, 2010). Human bone density and bone turnover remain relatively stable from the end of puberty to menopause (Adami, et al., 2010). However, both skeletal size and mass grow progressively during the period of sexual development (Xu et al., 2011). Changes in levels of growth hormone, Insulin-like Growth Factor and sex hormones play crucial roles in affecting bone modeling, bone maturation and bone mass accumulation and consolidation (Saggese, Baroncelli & Bertelloni, 2002).

1.1.5.1.1 Growth Hormone

Growth hormone (GH) is a single chain peptide which plays a critical role in regulating bone growth and bone remodeling. It regulates osteoblast cell proliferation and differentiation by regulating the GH receptors on osteoblasts (Adami et al., 2010). GH positively affects bone growth during the pre-pubertal period (Libanati, et al., 1999), and is critical for accretion of bone mass during adolescence and maintenance of bone mass during adulthood (Adami et al., 2010).

The production of GH is increased during sexual development. During pre-puberty, GH primarily promotes linear bone growth. The peak level of GH production coincides with peak height velocity (MacKelvie, Khan, & McKay, 2002;Walsh, Fatayerji, & Eastell, 2010). Merimee and colleagues (1991) examined the relationship between height velocity and GH and found that

height velocity correlated strongly with GH in girls aged 10-16 years old. The effects of GH are mainly mediated via insulin-like growth factor I (Libanati, et al., 1999). It is well accepted that the GH/IGF-1 axis is an essential regulator of bone growth (Yakar, et al., 2002; Courtland et al., 2011).

1.1.5.1.2 Insulin-like Growth Factor-I

In addition to GH, Insulin-like Growth Factor (IGF-1), a growth-promoting polypeptide, is also critical for bone development because many skeletal cell proliferation and differentiation pathways that are regulated by growth hormone are mediated by (IGF-1). Studies have shown that IGF decreases collagen degradation while increasing bone matrix formation and osteoblast recruitment. Hence, IGF-1 plays an important role in bone anabolic regulation (Wallander et al., 2006).

Approximately 75% of serum IGFs are composed of three parts, an IGF molecule, an IGF binding protein-3 (IGFBP-3), and an acid labile subunit (ALS). The levels of circulating IGF-1 are regulated by GH. IGF-1 regulates bone growth in endocrine, paracrine, and/or autocrine manners, and the local IGF-1 is considered more important than circulating IGF-1 in chondrogenesis regulation in animal models (Yakar, et al., 2002; Xu, et al., 2011). Studies using mouse models with liver-specific IGF-1 inactivated showed a 75% reduction in circulating IGF, but only a small deficit in bone growth (Sjogren et al., 1999; Yakar et al., 1999). Levels of IGF-1 increase rapidly during childhood and reach their peak in early adulthood (Xu, et al., 2011; Lofqvist et al., 2005). IGF binding protein-3 (IGFBP-3) is the major binding protein of IGF-1

which functions in regulating bone metabolism (Kanbur, Derman, & Kınık, 2005; Yakar, et al., 2002). Both IGF-1 and IGFBP-3 serum levels are increased during puberty (Yakar et al., 2002; Adami et al., 2010; Wallander et al., 2006).

1.1.5.1.3 Sex Hormones

Sex steroids are increased during puberty and play a major role in bone growth and mineralization (Libanati, et al., 1999). It is well accepted that estrogens play a key role in the accumulation and maintenance of bone mass. Large numbers of studies have shown that estrogen reduces bone resorption via regulating the gene expression, size, and apoptosis of osteoblasts and osteoclasts (Bradford, Gerace, Roland & Chrzan, 2010; Imai et al., 2009; Yilmaz et al., 2005). Yilmaz et al., (2005) reported that increasing levels of estrogen may be involved in promoting linear growth and skeletal mineralization in girls, and estrogen has the greatest positive effect on bone mineral accumulation compared to other hormones in children during puberty.

The effects of levels of serum testosterone and estradiol at different pubertal stages in girls have been correlated with increased BMD values. Before menarche, testosterone has a strong independent effect on bone linear growth. Estradiol also stimulates linear bone growth during the pre-menarche period, but inhibits longitudinal growth post-menarche (Xu et al., 2011).

In adolescence, gonadal sex steroids are key factors that promote bone growth and modeling. Buchanan et al. (2009) examined the role of testosterone, androstenedione, and estradiol on peak

trabecular bone density of women ages18-22 years who had different levels of physical activity. The researchers found that androgens and estrogen significantly affect trabecular BMD in sedentary women (Buchanan et al., 2009).

1.1.5.2 Sexual Maturity Assessment: Tanner Stage

The concerted regulation of the hypothalamic-pituitary-ovarian system along with other endocrine systems determines the timing of puberty (Xu et al., 2011). Due to the influences of hormonal changes, late childhood and early adolescence is characterized by physiological and behavioral changes (Duke, Litt & Gross, 1980). During this time period, chronological age does not adequately present the changes that are occurring (MacKelvie, Khan, & McKay, 2002). One study reported that the development of children with the same chronological age could differ by as much as six years (Malina & Bouchard, 1991). Thus, it is important to include both age and maturity of the subjects (MacKelvie, Khan, & McKay, 2002).

Among various sexual maturity assessment methods, self-reported levels of sexual maturation using Tanner stage photographs are thought to be highly accurate and non-invasive (Duke, Litt & Gross, 1980;MacKelvie, Khan, & McKay, 2002). Children in Tanner stage I are classified as prepubertal; Tanner stages 2 and 3 are considered to be in an early pubertal stage. Adolescents in Tanner stages 4 are defined as in late stages of puberty, and in Tanner stage 5 are thought to be post-pubertal (MacKelvie, Khan, & McKay, 2002).

Yilmaz and colleagues (2005) reported that the peak BMD value occurred at Tanner stage IV, and the bone mass increase in girls was rapid between 11 and 15 years old. This indicates that the greatest increase of bone mass acquisition coincides with pubertal development in children.

1.2 Diet and Nutrition

1.2.1 Children's Overall Diet in the U.S.

While general agreement has been reached that 60-80% of variation of peak bone mass depends on genetic factors, modifiable factors such as diet and physical activity still play important roles in bone development (Rizzoli, Bianchi, Garabedian, McKay & Moreno, 2010). During childhood and adolescence, adequate dietary intakes facilitate reaching a higher peak bone mass. In adulthood, proper diet helps to slow down loss of bone mineral content and further reduce the risk of fracture. An epidemiological study showed that an increase in peak bone mass by 10% could lower the risk of bone fractures after menopause by 50% (Simmonds, 2007). Therefore, is important to have an adequate diet and adequate physical activity for bone health, particularly during adolescence, because bone is more responsive to nutrients and mechanical stimuli for bone development during this period compared to adulthood (Bonjour, 2011).

Food groups such as dietary protein, fat, fruits and vegetables could play key roles in bone growth and bone mass maintenance (Levis & Lagari, 2012). A longitudinal study conducted with 292 children ages 3 to 7 showed that increased intakes of dark-green and deep-yellow vegetables, as well as limited fried food intakes, can result in greater rates of bone mass accrual (Wosje et al., 2010).

However, the diets of children and adolescents are not meeting recommendations. The trends show increases in total energy intake, portion sizes, and energy-dense foods while fruit and vegetable intakes are low (Piernas & Popkin, 2011). In 2009-2010, the average intake of calories from solid fat and added sugar (SoFAS) among U.S. children was 33% while the recommended level ranged from 8%-19% and depended on the total energy requirements (Dietary guidelines for Americans, 2010). Reedy and Krebs-Smith (2010) found that milk, sugar-sweetened beverages (SSBs), pizza, and French fries are the top food sources of SoFAS, which children can easily obtain from fast-food restaurants, schools and stores (Serrano & Jedda, 2009; Harris, Schwarts & Brownell, 2010). Unlike the SoFAS, fruit and vegetable intakes in US children and adolescents are below recommended levels (Faith, Dennison, Edmunds & Stratton, 2006; Guenther, Dodd, Reddy & Krebs-Smith, 2006). The correlates of fruit and vegetable intake of 6513 children and adolescents age 2-18 were analyzed using 1999-2002 NHANES data. The study found that French fries were the leading vegetable source, which accounted for more than 28% of the vegetable intake. The leading source of fruit was 100% fruit juice (Lorson, Melgar-Quinonez & Taylor, 2009).

1.2.2 Nutrition

1.2.2.1 Vitamins

1.2.2.1.1 Vitamin D

Vitamin D plays an essential role in calcium and phosphate metabolism in bone, the small intestine, and the kidneys. The Recommended Dietary Allowance (RDA) of Vitamin D is 15 μg (Dietary Reference Intakes, 2010). Vitamin D is converted from vitamin D_3, which can be consumed through diet or synthesized from previtamin D_3 and its precursor 7-dehydrocholesterol

in skin under sunlight exposure (Rajakumar, Greenspan, Thomas & Holick, 2007). Vitamin D_3 is converted to 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver, and further converted to the active form of vitamin D, calcitriol ($25(OH)_2D_3$) in the kidneys (Stern, Philips & Mavreas, 1980).

In skeletal tissue, calcitriol functions as a calcium regulator though the vitamin D receptor (VDR) mediated pathway. Calcitriol binds to VDR on the osteoblasts, which stimulates Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) expression in osteoblasts, which further induces osteoclastogenesis and produces macrophage colony-stimulating factor (M-CSF). M-CSF stimulates osteoclast precursors to proliferate and differentiate into mature multinucleated osteoclasts that are capable of bone resorption. This results in an increasing rate of bone resorption leading to elevated level of serum calcium (Suda et al., 1999; Adamopoulos, et al., 2006).

In the small intestine, calcitriol plays a key role in facilitating calcium absorption. In transcellular pathways, calcium is transported through the apical calcium channel transient receptor potential vanilloid type 6 (TRPV6) and is transferred to the basolateral membrane by a protein called calbindin and extruded by plasma membrane pump PMCA1b (Christakos, Dhawan, Porta, Mady & Seth, 2011). Calcitriol increases TRPV6 mRNA levels and calbindin-D_{9k} expression, thus promoting calcium absorption (Song et al., 2003). Calcitriol stimulates paracellular calcium transport, in which calcium is transported through tight junctions connecting apical and basolateral sides of enterocytes. Calcitriol is thought to increase paracellular calcium transport by

increasing junction permeability (Fujita H. et al, 2008; Christakos, Dhawan, Porta, Mady & Seth, 2011).

For vitamin D, 600 International Units per day is needed for people of all genders and ages across North America (Dietary Reference Intakes for Calcium and Vitamin D, 2010). Insufficient vitamin D levels in the body can cause bone problems throughout an individual's lifespan. Inadequate serum vitamin levels (25HD < 20ng/mL) are highly associated with subclinical problems such as low level of gastrointestinal calcium absorption and bone mineralization as well as a lag in bone development. Severe vitamin D deficiency (25HD <10 ng/mL serum) could cause rickets and osteomalacia (Levis & Lagari, 2012). Rickets are found in children and adolescents when inadequate mineralization occurs at the expanding growth plate cartilage, and deforming of bones occurs (Winzenbergy & Jones, 2013; Levis & Lagari, 2012).

Researchers investigated the relationship between vitamin D and the acquisition of bone mass in 193 girls aged 10-12 years in Finland (Cheng et al., 2003). Participants' dietary information was collected through a 3-day food-intake diary. BMC, aBMD of the whole body, femoral neck, total femur, and lumbar spine were measured by Dual-energy X-ray absorptiometry (DXA). The volumetric bone mineral density (vBMD) of the distal radius and the tibia shaft were measured by pQCT. The researchers found that participants deficient in vitamin D had higher levels of PTH and lower cortical vBMD of the distal radius and tibial shaft (Cheng et al., 2003).

Another study recruited 211 healthy European girls aged 11 to 17 years to investigate the relationship between vitamin D levels and bone mineralization (Esterie et al., 2010). Their dietary information was obtained by a 7-day food recall. The participants' BMDs were measured

by DXA. The researchers found that lower plasma vitamin D levels were associated with lower tibial aBMD, although the participants had adequate calcium intake. The study also suggests that low vitamin D levels (less than 40nmol/L serum) may negatively impact lumbar spine mineralization when the participants also have low intakes of calcium (less than 600mg/day) (Esterie et al., 2010).

1.2.2.1.2 Vitamin C

The DRI for vitamin C for females aged 9-13 is 45 mg/d (Dietary Reference Intake Tables and Application, 2010). Vitamin C modifies collagen synthesis by catalyzing posttranslational hydroxylation of proline and lysine residues, which are important for stabilizing collagen triple helixes. It functions as a precursor of bone matrix mineralization to induce osteoblast differentiation and increase the rate of osteoclast formation (Morton, Barrett-Connor & Scheider, 2001). Studies have shown that vitamin C promotes alkaline phosphatase activity which is essential for type I collagen formation and bone mineralization (Chan, Lamande, Cole & Bateman, 1990). In animal studies, severe vitamin C deficiency has been found to cause scurvy, which was associated with decreasing levels of BMD and BMC (Ahmadieh & Arabi, 2011).

Laudermilk et al. (2012) examined the relationship between vitamin C intake and bone structure and strength in 4th and 6th grade girls. The areal BMD and BMC of the whole-body, total hip and lumbar spine 2-4 were measured by DXA. pQCT was used to assess the volumetric BMD, strength-strain index (SSI, mm^3), and bone geometry which includes trabecular and cortical density, area, endosteal and periosteal circumferences. DXA data revealed a positive effect of

vitamin C intake that was approaching significance in the total hip (p= 0.07) and lumbar spine (p= 0.09) among 4th grade girls. Results from pQCT indicated a significant positive relationship between vitamin C intake and trabecular geometry and cortical strength in 4th grade girls. No significant association was found among 6th grade girls. Noteworthy, the vitamin C intakes were more than twice of the RDA in the participants (Laudermilk et al., 2012).

1.2.2.1.2 Vitamin K

Vitamin K has two naturally occurring forms, vitamin K1 and K2. Vitamin K1/phylloquinone/phytonadione is rich in some green leafy vegetables and plant oils. Vitamin K2/menaquinones-n is synthesized by colon bacteria. The vitamin K1 DRI for females aged 9-13 is 60 μg/d (Dietary Reference Intake Tables and Application, 2010). Though recent studies have reported that vitamin K2 may be more biologically active than vitamin K1, the Institute of Medicine has not provided DRI values for vitamin K2 due to lack of evidence (Hamidi, Gajic-Veljanoski & Cheung, 2013).

Vitamin K is best known for its important role in blood coagulation. It is also thought that vitamin K and vitamin D facilitate osteocalcin synthesis. Vitamin D stimulates the synthesis of osteocalcin by promoting gene transcription, while vitamin K facilitates the post-translational conversion of glutamyl to γ-carboxyglutamyl (Gla) residues, which has been found in bone (Szulc et al., 1993). The circulating concentration of under-ɣ-carboxylated osteocalcin (ucOC) is thought to be a marker of vitamin K nutritional status (Sokoll & Sadowski, 1996). The marker

has been found to be a predictor of hip fracture risk in adults (Szulc et al., 1993; Szulc et al., 1996; Booth et al., 2000), and bone mineral content in young girls (O'Connor et al., 2007).

A double-blind, placebo-controlled study evaluated the relationship between vitamin K and bone health among 223 healthy Danish girl aged 11-12 years (O'Connor et al., 2010). The researchers found that better vitamin K status was associated with decreased bone turnover and higher total body and lumbar spine bone mineral content (O'Connor et al., 2010).

1.2.2.1.3 Vitamin A

The role of vitamin A intake on bone health is historical yet controversial (Scheven & Hamilton, 1990; Togari et al., 1991). Vitamin A is obtained from animal sources and fortified foods in form of retinoic acid, and as a provitamin from plant-derived foods (Penniston & Tanumihardjo, 2006). The RDA for vitamin A is given as mcg of retinol activity equivalents (RAE) to account for different bioactivities of retinol and provitamin A carotenoids. The RDA for girls 9-13 years old is 600 mcg RAE (Vitamin A fact sheet for health professionals, National Institute of Health).

Retinoic acid, the preformed vitamin A, has been shown to inhibit osteoblast activity and promote osteoclast formation (Scheven & Hamilton, 1990; Togari et al., 1991), and evidence for a negative effect of excess retinol on bone has been reported both from laboratory animal models and human studies (Lind et al, 2011, Kneissel, Studer, Cortesi & Susa, 2005). Promislow et al. (2002) reported an inverse U-shaped association between retinol intake and aBMD in

postmenopausal women. Different from retinol, the carotenoids are a plant sources of vitamin A, which are antioxidants that may play an important role in preventing oxidative stress-related osteoclasto-genesis and bone resorption (Sugiura et al. 2011). With fruits and vegetables being their richest source, carotenoids are also considered to be a biomarker that reflects healthy dietary habits which can benefit bone health (Tanumihardjo, 2013). Therefore, carotenoids are considered to have a beneficial role in preventing oxidative stress-related osteoclasto-genesis and bone resorption (Sugiura et al. 2011).

1.2.2.2 Minerals

1.2.2.2.1 Calcium

A general agreement has been reached on the significant positive impact that calcium has on skeletal health (Levis & Lagari, 2012). According to the DRIs, 1300 mg per day of calcium is adequate to meet the needs of more than 98% of children aged 9-13 years old (Dietary Reference Intakes for Calcium and Vitamin D, 2010). Calcium is the most important nutrient for bone. It plays a key role in bone growth and formation. The adult human body contains about 1200 g of calcium, which corresponds to about 1-2 % of body weight and 32% of bone mineral content. Nearly all the calcium in the human body is found in mineralized tissues, such as bones and teeth, in the form of calcium phosphate to provide rigidity and structure. The rest of the calcium is found in body fluids, muscle, and other tissues. It also plays a role in mediating vascular contraction and vasodilation, muscle contraction, nerve transmission and glandular secretion (Cashman, 2002).

Calcium serum concentration levels are controlled within a small range (1.1-1.3 mmol/l). Therefore calcium absorption, excretion and secretion are tightly regulated by multiple calciotropic hormones including PTH, calcitriol, and calcitonin. These hormones regulate serum calcium levels by targeting the kidneys, small intestine, and bone (Cashman, 2002). The secretion of these hormones is stimulated by plasma calcium levels. PTH and calcitriol increase plasma calcium levels, while calcitonin reduces calcium levels. If the serum calcium levels decrease due to inadequate calcium intake, PTH is released to return blood calcium levels to the normal range by stimulating bone resorption. Thus, long-term calcium intake deficiency leads to negative bone mineral balance (Weaver, 2014).

Calcium is especially important for bone growth and development during adolescence. Approximately 150 mg of calcium is accumulated in bone tissue daily until individuals reach their early twenties. Subsequently, the body reaches calcium equilibrium. Peak bone mineral density is achieved in early adulthood. Bone mass remains relatively stable from approximately 20 to 40 years of age, and then is gradually lost in both sexes (Rentice, 1997). Decreased production of estrogen in women at menopause accelerates bone loss (Compston, 1993). The condition of negative calcium balance increases the risk of osteoporosis, especially in women. Adequate calcium intake during childhood and adolescence is critical for increased peak bone mass, thus reducing the risk of osteoporosis later in life (Bianchi, 2007).

To evaluate the effects of calcium supplements on BMD of children, a meta-analysis examined 19 randomized control trials from 1992-1995 that included 2859 healthy children aged 3-18.

Calcium supplements used in the studies ranged from 300-1200 mg/d. Forms of supplements included calcium carbonate, calcium citrate malate and calcium phosphate, calcium lactate gluconate, calcium phosphate milk extract, or milk minerals. The intervention period for all the studies was at least 3 months, and the follow-up time for BMD measurement was at least six months. Sites of interest for BMD were femoral neck, lumbar spine, total body, and upper limb. The reviewers concluded that no significant difference was found for BMD at femoral neck or lumbar spine between the children who consumed calcium supplements compared to controls. Very small differences were found in upper limb BMD, which were equivalent to an approximately 1.7 % greater increase in BMD in the supplemented group than the control group. The small increase in BMD of upper limb bone is unlikely to significantly reduce the risk of fracture clinically (Winzenberg, Shaw, Fryer & Jones, 2006).

In addition to calcium supplements, dietary calcium intake and bone health has also been evaluated by multiple studies. However, conflicting results have been reported. Feskanich et al (2013) examined the effect of milk consumption during adolescence on the risk of hip fractures later in life. The prospective cohort study followed 96,000 white postmenopausal women from the Nurses' Healthy Study and men aged 50 years or older from the Health Professionals Follow-up Study in the US for over 22 years. A total of 1226 hip fractures were reported in 490 participants. Surprisingly, the results indicated that each additional glass of milk per day during teenage years was associated with a significant 9% higher risk of hip fracture in men (RR = 1.09; 95% CI, 1.01-1.17). However, the association was attenuated when height was included (RR = 1.06; 95% CI, 0.98-1.14). No significant association was found between milk consumption during teenage years and hip fractures in women.

1.2.2.2.2 Magnesium

Bone is the largest reservoir of magnesium in human body. About 60% of magnesium in the body is stored in bone tissue. The RDA for Magnesium is 310 mg/d (Dietary Reference Intakes Tables and Application, 2010). Magnesium functions to prevent forming of brittle bone by decreasing the size of hydroxyapatite crystal. Magnesium also plays a role in transporting potassium and calcium ions, influences bone formation, osteoblast and osteoclast functions, and facilitates calcium metabolism (Palacios, 2006). Some studies have shown that high calcium intake may negatively affect magnesium utilization. Andon and colleagues (1996) evaluated the effect of magnesium intake in adolescent females consuming a low or high calcium diet reported that alteration in magnesium utilization should not be anticipated in female adolescents who follow the current recommended dietary allowance for magnesium.

1.2.2.2.3 Phosphorus

The RDA for Phosphorus is 700 mg/d (Dietary Reference Intakes Tables and Application, 2010). At least 80% of the phosphorus in the body is stored in the bones and teeth. It is thought that phosphorus depletion causes impaired bone mineralization. Acute dietary phosphorous deficiency leads to skeletal demineralization even before the serum phosphorus levels decrease. No direct evidence shows that phosphorus deficiency increases the risk of osteoporosis in healthy individuals. More concern is on the impact of high dietary phosphorus on bone, which has a deleterious effect on bone through increased parathyroid hormone (PTH) secretion that promotes bone resorption (Palacios, 2006; Takeda, Yamamoto, Yamanaka-Okumura & Taketani, 2012).

1.2.2.2.4 Fluoride

In contrast to magnesium, fluoride increases the hydroxyapatite crystal size by forming relatively more insoluble fluoroapatite. The RDA of Fluoride is set to be 2 mg/d for females 9-13 years old (Dietary Reference Intakes Tables and Application, 2010). Fluoride is used to prevent bone fracture because it promotes osteoblast activity. Fluoridation of drinking water is being used to improve dental health. However, it is thought that high intake of fluoride (>50 mg/d) might cause brittle bones due to the large size of hydroxyapatite (Lehmann, 1998). Moderate levels of fluoride intake (11-20 mg/d) are reported to decrease vertebral fracture risk and increase spine and femoral neck bone mass density (Meunier, 1998; Palacios, 2006).

1.2.2.2.5 Zinc

Zinc is required for osteoblast activity, collagen generation and alkaline phosphatase activity. Studies have found that low serum zinc concentration and high urinary zinc excretion are related to osteoporosis (Palacios, 2006). A two-year longitudinal study reported that calcium supplements with zinc, copper, and manganese led to a greater increase in bone mass compared with calcium supplements alone (De Jong, et al., 2001). The RDA of zinc is 8mg/d for females 9-13 years old (Dietary Reference Intakes Tables and Application, 2010).

A recent study examining the relationship between zinc intake and bone in girls in 4th grade and 6th grade reported that zinc was positively associated with femur and tibia cortical density in 4th grade girls. No significant association was found among 6th grade girls. (Laudermilk et al., 2012).

1.2.3 Macronutrients

1.2.3.1 Protein

Protein composes approximately 50% of bone volume and 30% of bone mass. The DRI for protein is 46 g/d for female adolescents (Dietary Reference Intakes, 2010). The skeletal protein matrix has multiple functions and undergoes turnover and remodeling continuously. A large percentage of the bone matrix collagen is released during remodeling and cannot be reused to generate new bone matrix (Heaney & Layman, 2008). Thus, dietary protein is essential for bone formation because amino acids are needed for synthesizing intracellular and extracellular bone proteins.

Protein also affects calcium and phosphate balance and bone metabolism by regulating the synthesis of IGF-1. IGF-1, stimulated by dietary protein, has a positive effect on bone mineral development through a dual renal action. IGF-1 promotes the synthesis of calcitriol, which will further induce the intestinal absorption of calcium and phosphate. In addition, calcitriol also stimulates calcium and phosphate resorption in the kidneys. Dietary protein deficiency could reduce the production of IGF-1, negatively affecting skeletal integrity (Thissen, Triest, Maes, Underwood & Ketelslegers, 1990).

Nevertheless, it has been reported that protein intake has indirect effects on bone through its influence on the overall dietary acid-base balance (FAO/WHO/UNU, 2007). Urinary calcium has been reported to increase with acid-forming food sources, because calcium is used to balance the endogenous acid synthesized from acid-forming foods (Spence & Weaver, 2003). Protein from animal sources in particular can cause increased levels of calcium excretion, thus influencing

calcium homeostasis and could lead to further chronic metabolic acidosis and osteoporosis (Heaney & Layman, 2008).

However, it is still debatable as to whether high protein intake could lead to high calciuria and negative calcium balance (Bonjour, 2005), or whether protein from plants is better for bone health than animal protein (Bonjour, 2005). Results from a meta-analysis showed that higher protein intake was associated with greater bone mass when calcium intake was sufficient. The researchers recommended further study to focus more on increased fruit and vegetable consumption rather than reducing animal protein intakes (Heaney & Layman, 2008).
Some studies raised the question that whether different levels of dietary protein intake in the normal range can affect skeletal development. It was found that that a protein intake within the normal range can positively affect skeletal growth. Thus, relatively high dietary protein consumption could promote bone mass accretion during childhood (Bonjour, Chevalley, Rizzoli & Ferrari, 2007).

1.2.3.2 Fats

1.2.3.2.1 Saturated Fat

Dietary fats are shown to have important effects on skeletal health in animal studies (Hoffman et al., 1999; Judex et al., 2000; Corwin, Hartman, Maczuga, & Graubard, 2006). Only a limited number of studies have examined the relationship between bone health and saturated fatty acid intake. In NHANES III, the relationship between BMD and fat intake was examined in 13572 participants. No significant association between total fat intake and BMD was found at the analyzed sites (femoral neck, intertrochanter, trochanter, and total hip). However, saturated fat

intake was negatively associated with BMD in the femoral neck, trochanter, and total hip of all participants. The negative associations were more significant in men than in women across all ages. Thus, total amount of saturated fat consumption may significantly affect bone health (Corwin, Hartman, Maczuga, & Graubard, 2006). However, it is also possible that the intake of saturated fat was coming from animal sources, resulting in higher blood acid levels as discussed in the previous section. No study was identified the relationship between saturated fatty acid intake and bone mass in children.

1.2.3.2.2 Unsaturated Fat

The polyunsaturated fatty acids (PUFAs), especially ω-3 and ω-6, have been reported to improve bone health through multiple mechanisms. First, essential fatty acids promote vitamin D-dependent calcium absorption through enhancing calcium absorption in the intestine and decreasing calcium excretion (Hay & Hassam, 1980; Orchard, Pan, Cheek, Ing, & Jackson, 2012). Second, FAs (ω-3, ω-6) have opposing effects on inflammatory cytokines (Kettler, 2001), and the pro-inflammatory cytokines are an important regulator of bone turnover and calcium balance (Orchard, Pan, Cheek, Ing, & Jackson, 2012). They regulate the NF-ϰB ligand (osteoprotegerin (OPG)/RANKL) ratio in bone (Hofbauer & Schoppet, 2004). While RANK enhances the formation and activation of osteoclasts, OPG inhibits RANKL from activating RANK. Therefore, a lower ratio of (OPG)/RANKL leads to greater bone resorption (Hofbauer & Schoppet, 2004; Orchard, Pan, Cheek, Ing, & Jackson, 2012). The ω-3 FAs have been suggested to reduce the level of the eicosanoid prostaglandin E_2 (PGE), which decreases OPG/RANKL levels. Fatty acids are shown to increase bone density by promoting vitamin D dependent

calcium absorption (Hay et al., 1980). A study in Sweden found a positive association between the serum concentration of fatty acids, DHA in particular, and BMD of 22 year old healthy men (Högström, Nordström & Nordström, 2007). An increased ratio of dietary ω-6 to ω-3 fatty acids was found to be significantly associated with lower BMD at the hip in a group of older, middle to upper-middle class, Caucasian residents of southern California (Weiss, Conner & Muhlen, 2005). Damsgaard et al. (2012) reported that no association was found between fish oil supplements, DHA status and bone mass in a cohort of healthy, slightly overweight adolescent boys aged 13-15 years old.

1.3 Physical Activity

While optimal nutrient and energy intakes are essential for bone health, exercise is also critical for building healthy and strong bone. Physical activity is one of the modifiable environmental factors that influences bone health. It accounts for approximately 17% of the variation in BMD (Boreham & McKay, 2011). Physical activity increases bone accretion during growth, thus reducing the risk of osteoporosis (Boreham & McKay, 2011).

On the cellular level of bone tissue, 95% of the bone cells consist of osteocytes. While the other three types of bone cells (osteoblasts, osteoclasts and bone lining cells) are located at the surface of bone, osteocytes are located inside of the skeleton matrix. The half-life of osteocytes is on average 25 years (Rochefor, Paliu & Benhamou, 2010), and the length can be altered by mechanical stimuli. The number of apoptotic osteocytes is decreased in response to mechanical force load on the bone through physical activity (Plotkin et al, 2005). It is thought that bone adapted to external mechanical stimuli better during growth than during other maturational

phases (Boreham & McKay, 2011), which is based on the assumption that mechanical force has its greatest effect on bone surfaces during the time period when it is covered with a larger proportion of osteoblasts (Robling et al, 2002).

A cohort study examined the effects of physical activity on bone mass in 4457 children aged 11 years old who participated the Avon Longitudinal Study of Parents and Children in the United Kingdom during 2003 and 2005. A positive association was found between BMD and physical activity among participants, adjusted for height, lean mass and fat mass (Tobias, Steer, Mattocks, Riddoch & Mess, 2006).

1.3.1 Timing of Physical Activity

Bone mineral accrual is strongly influenced by hormones and growth factors; thus the rate of bone mineral acquisition differs during phases of the lifespan. Studies reported that before puberty or during early puberty, bone growth and mineral accrual are most responsive to physical activity and have greater capacity to adapt to mechanical stimulation (Hind & Burrow, 2007; Bass. et al. 1998; Ishikawa, Kim, Kang & Morgan, 2013). In a review of controlled trials on weight-bearing exercise and bone mineral accrual in children and adolescents, 6 out of 9 studies found positive effects from physical activity on bone mass among prepubertal children, ranging from 0.9% to 4.9% over 6 months (Hind & Burrows, 2007). All studies showed significant positive effects from physical activity on bone mass, which ranged from 1.1% to 5.5% over 6 months; 2 out of 5 studies reported positive effects from physical activity on bone mass of pubertal adolescents, ranging from 0.3% to 1.9% over 6 months (Hind & Burrows, 2007).

1.3.2 Type of Physical Activity

It is thought that different types of exercise improve bone strength in different ways. Weight bearing activities stimulate bone growth and mineral accrual by increasing mechanical loading on bone (Huang et al., 2003). Studies have found that weight-bearing or impact-loading exercise such as stepping and running result in significantly greater bone accretion compared with non-weight-bearing exercise, such as swimming (Huang et al., 2003; Greene, Naughton, Bradshaw, Moresi & Ducher, 2012).

The stimulation of bone accretion via impact loading is also thought to be site specific. A study investigated the inter-individual differences in response to mechanical loading in bone by examining the BMD of 91 female tennis players (aged 7-17 year) and 58 female controls (Hapasaalo et al., 1998). In each Tanner stage, BMD between the playing and nonplaying arms of the tennis players was significantly different, while still significant but much smaller in between the two arms of non-tennis players. The results showed the side- to side differences in arm BMD, thus demonstrating mechanical loadings are site specific (Hapasaalo et al., 1998).

1.3.3 Duration and Frequency of Physical Activity

Turner and Robling (2005) reported that bone cells' sensitivity to mechanical stimulation decreases after prolonged physical activity in rats. The unloading period can enhance bone formation which is stimulated during the loading period. Osteogenic responses can be increased by resting between skeletal loading sessions (Turner & Robling, 2005). Mechanical loading was found to be more effective in promoting bone biomechanical and structural properties if

exercising loads are discrete and separated by recovery periods, rather than applied as one long training session in rats (Robling et al, 2002). Robling et al. (2002) used animal models to evaluate the desensitization and resensitization of bone cells to mechanical loading. Only the right ulnas of the rats in the two experimental groups were subjected to 360 compressive loads applied axially per day, 3 days per week, and 16 weeks in total. The only differences in the two groups were the duration and frequency of the load cycles. The 360 load cycles were applied to the first group in a single, uninterrupted session. The second group was administered the 360 cycles in four discrete bouts, 90 cycles in each bout, and 3 hours of recovery period between bouts. The results of the study showed that the loaded/right ulnas of both groups had small gains in aBMD and BMC and large improvements in bone force and energy to failure, with greater benefits in the second group. The results led to a conclusion that mechanical loading that is divided into discrete bouts and separated by recovery periods can achieve more beneficial effects on bone mass and strength (Robling, Hinant, Burr & Turner, 2002).

1.4 Bone Mineral Content/Density and Dietary Intake Assessment

1.4.1 Bone Mass Measurements

From pre-puberty to adolescence, bones are growing in length, width, and thickness, which results in a dramatic acceleration in bone mass accrual. BMD and BMC are important measurements to assess changes in bone mass when bones are growing (Mazess, Barden, Bisek & Hanson, 1990; Rizzoli et al., 2010).

1.4.2 Non-invasive Bone Assessment Tools: DXA and pQCT

The two most common methods for pediatric bone assessment are dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT). DXA is used to measure BMC and aBMD. DXA can rapidly measure large regions of interest, has high precision and accuracy as well as low radiation exposure. and its measurement is strongly correlated with risk of fracture (Dowthwaite, Flowers & Scerpella, 2011; Mazess, Barden, Bisek & Hanson, 1990; Rizzoli et al., 2010).

pQCT measures skeletal tissue three dimensionally and it can distinguish between trabecular bone and cortical bone. Therefore pQCT is considered an ideal bone measurement method. However, compared to DXA, many research centers do not have access to pQCT. In addition, pQCT measurement has relatively greater movement and positional variation at both within and between individuals, which makes it less accurate for children and adolescents (Dowthwaite, Flowers & Scerpella, 2011).

Compared with pQCT, DXA has lower inter and intra individual variation due to lower sensitivity to movement, which increases the scan quality, particularly among children. A major limitation of DXA is that it only measures skeletal tissue in two dimensions. Therefore though DXA measurement can be used as a good fracture risk indictor, it does not evaluate true volumetric bone density or skeletal geometry. Additionally, DXA does not distinguish between trabecular and cortical bone. Vertebral trabecular bone are at higher risk of fracture (Dowthwaite, Rosenbaum & Scerpella, 2011).

To improve DXA measurement of the spine, a posteroanterior (PA) DXA scan is paired with supine lateral scan (LAT). LAT scans are used to isolate the vertebral body (trabecular tissue) from the posterior element (cortical tissue) of the vertebra. The combination of PA and LAT scans measure the skeletal tissue three dimensionally, which represents by the bone mineral apparent density (BMAD) (Dowthwaite, Rosenbaum & Scerpella, 2011).

1.4.3 BMC

BMC represents the bone mineral content in grams. Postero-anterior dual energy X-ray absorptiometry (PA DXA) measures BMC of the vertebral body and posterior elements. Supine lateral scans (LAT DXA) measure can isolate BMC from the vertebral body. Paired PA and LAT scans measure bone content in three dimensions: skeletal width, depth, and height (Dowthwaite, Rosenbaum & Scerpella, 2011). This allows for superior estimation of three-dimensional bone structure and strength indices.

1.4.3.1 aBMD

Areal BMD (aBMD) divides the bone mineral content by the projected bone scanned area in grams per square centimeter.

1.4.3.2 BMAD

BMAD is a measurement of volumetric bone density calculated by dividing bone mineral content by the bone volume in grams per cubic centimeter (Mazess, Barden, Bisek & Hanson, 1990).

1.4.2 Dietary Intake

Dietary intake assessment is conducted by collecting information on quantity and frequency of food consumption to obtain the energy and nutrient intakes. The selection of a dietary intake estimation method depends on the study objective, primary foods or nutrients of interest, characteristics of the population, study time frame, level of accuracy, and available resources. Dietary intake information on the individual level can be collected by short-term and long-term methods depending on the focus of the study. Short-term instruments focus on current intake and can include 24-hour dietary recalls and food records. Long-term methods, such as food frequency questionnaires and diet histories, collect usual diet over a longer period of time, usually months or years (Biró et al., 2002).

1.4.2.1 The 24h Food Recall

This method can be conducted face-to-face or over the phone. The respondents are asked to recall the type and quantity of food and beverages consumed during the preceding 24 hours. Interviewers need to be well trained and familiar with the dietary habits of the respondent to collect detailed and complete information. The advantage of the 24h recall is that the format of the interview is open-ended, and thus does not alter respondents' reported dietary patterns (Biró et al., 2002). The disadvantage of this method is that the respondents many not be able to remember what they consumed the preceding day during the recall process. They also tend to forget what they consumed or leave out items that are considered unimportant. Additionally, the portion size estimation is also challenging, as it needs to be accurately estimated and remembered (Thompson, Subar, Loria, Reedy & Baranowski, 2010).

The multiple pass 24 hr recall is a dietary assessment method developed by the USDA. This method guides the respondents to recall the food consumed during the past 24 h period in multiple passes to improve the recall accuracy. Respondents recall food intake without interruption during the first pass. Specific questions are asked by the data collector during the second pass according to the report in pass 1. The respondents review the reported food intakes chronologically to help in recalling additional food intakes during or between meals (Raper et al., 2004).

1.4.2.2 Dietary Records

Dietary records require respondents to record the amount of food consumption by weighing, estimating using utensils, or comparing to food models. The recording needs to be done at the time of consumption. The respondents need to be well trained to describe the necessary details of their diets. The dietary record requires high level of participation of the recorders, thus it might alter the dietary behaviors and cause fatigue over time (Biró et al., 2002). Same as 24-hour recall, the dietary records should not be used to assess long-term dietary intake.

1.4.2.3 Food Frequency Questionnaire (FFQ)

Food Frequency Questionnaires were developed to estimate the usual food intake of individuals. This is a relatively inexpensive tool to collect data from large numbers of respondents in a short amount of time. FFQ causes lower respondent burden compared to dietary records (Vereecken, Covents & Maes, 2010). FFQ can be used to easily compare food items with different levels of intakes. The disadvantages of this method are it depends on the memory of food intake in the

past, and the respondents need to be familiar with the standard portion sizes to report the correct portion sizes (Biró et al., 2002).

1.4.2.3.1 Youth/Adolescent Food Frequency Questionnaire

The Youth/Adolescent Food Frequency Questionnaire is a self-administered semi quantitative questionnaire that was developed by the Harvard School of Public Health to assess the dietary intakes of children and adolescents. This instrument was found to be accurate, reproducible and relatively inexpensive for repeated measures in the targeted age group (Rockett, et al., 1997). The YAQ was designed for youths 9 to 18 years old (Rockett, 2005). A total of 145 foods are included in the questionnaire, and each food has a standard serving size, or natural portions (eg: bread=slice). The eight frequency categories are ranged from "never or less than once per month" to "five+ per day." The total nutrient score for each item is calculated by multiplying the nutrient content of food by its corresponding frequency score (Rockett & Colditz, 1997). The validity of the YAQ was evaluated by comparing the average of two YAQs to three 24 hour recalls conducted from 261 youth and adolescents aged 9-18 years. The average energy for YAQ was higher but within 1% of the recalls. The means of all nutrients of the two methods were differed within 20%, with the exception of vitamin A, carotene, and alcohol (Rockett & Colditz, 1997).

1.5 Study Aims

Low bone mineral acquisition during childhood and adolescence increases the risk of osteoporosis and fracture later in life. Nutrition is one of the important modifiable factors which helps individuals to achieve adequate bone mineral acquisition and prevent osteoporosis. The

roles of nutritional factors, such as protein, calcium and vitamin D, in bone health have been well studied. However, there are still controversial results on the effects of energy, carbohydrate, protein, fat, vitamin C, vitamin K, phosphorous, potassium, magnesium and zinc on bone development, maintenance and fracture.

In addition to diet, physical activity, particularly weight-loading types, can promote bone mineral acquisition. Due to the strong influence of sexual maturity associated hormonal changes on bone mineral mass, using pre-pubertal females as a study population could help eliminate confounding factors. The objectives of this study are:

- To examine the relationship between key nutrient intakes (total intake and dietary alone without supplement) and bone outcomes of the third lumbar vertebra of pre-pubertal girls when accounting for the effects of age, height, and physical activity.
- To examine the relationship between physical activity and bone outcomes of the third lumbar vertebra of pre-pubertal girls when taking the effects of age, height, and each focal nutrient into account.

2. Manuscript

The Relationship between Diet, Physical Activity and

Dual Plane Dual-Energy X-Ray Absorptiometry Bone Outcomes in Pre-pubertal Girls

ABSTRACT

Osteoporotic fractures are a leading cause of morbidity in the United States. Bone mass levels during childhood play a key role in determining peak bone mass and hence the risk of

osteoporosis in later life. The aim of the current study was to evaluate how key nutrients and organized, non-aquatic physical activity affect bone outcomes in pre-pubertal girls using dual plane dual- energy X-ray absorptiometry (DXA). This cross-sectional analysis used a subset of participants (n=50) from a longitudinal study of bone growth in relation to physical activity. Dietary data were collected using the Youth/Adolescent Questionnaire (YAQ, 1995) and organized activity was recorded semi-annually to yield annual means (hours per week). Paired postero-anterior (PA) and supine lateral lumbar spine (LAT) DXA scans provided L3 PA areal bone mineral density (PABMD), PA vertebral width (PAWIDTH), bone mineral content (PABMC, LATBMC), LAT vertebral height (LATHEIGHT), LAT vertebral depth (LATDEPTH). Vertebral bone mineral apparent density (PALATBMAD), volume (PALATV), strength (PALATIBS), and fracture risk index (FRI) were calculated from paired PA bone width and lateral measures of bone geometry and mass.

Multiple linear regression was used to analyze the association between key nutrients, physical activity, and bone outcomes. We found significant, negative correlations between carbohydrate and fiber intakes with bone outcomes. After accounting for age, height, and activity, focal nutrients were not significant factors in bone outcomes. Physical activity was positively associated with PABMD, PABMC, PAWIDTH, LATBMC, PALATIBS after adjusting for age, height, and all key nutrients. We found physical activity may play a stronger role than nutrient intakes in influencing bone mass density, geometry and strength in well-nourished pre-pubertal girls.

INTRODUCTION

Osteoporosis is one of the leading causes of morbidity for women (Lippuner, Johansson, Kanis, & Rizzoli, 2009). Peak bone mass is negatively associated with the risk of osteoporosis, and a large percentage of peak bone mass is accumulated during childhood and adolescence. Hence, bone mass accretion during childhood and adolescence plays a key role in influencing the risk of osteoporosis in later life (Huncharek, Muscat, & Kupelnick, 2008). While 60-80% of variation in peak bone mass is dependent on genetic factors, modifiable factors such as diet and physical activity still play important roles in bone development (Rizzoli, Bianchi, Garabedian, McKay & Moreno, 2010).

Researchers agree that calcium and vitamin D are key nutrients needed for bone growth and maintenance in children and adults (Levis & Lagari, 2012; Brannon et al, 2008; Rafferty et al, 2008). Past studies have demonstrated that calcium and vitamin D from both dietary sources and supplements have been positively associated with bone mineral density (BMD)(Cheng et al. 2005; Cheng et al.2003; Esterie et al., 2010) and lower bone fracture risk (Feskanich et al 2013).

Between 2007 and 2010, the mean total calcium intake and the intake of calcium without supplementation among U.S. girls aged 9-13 years old was 988 ± 47.1 mg/d and 969 ± 44 mg/d, respectively. These intakes are lower than the recommended dietary allowance (RDA). The total vitamin D intake (308 ± 40 IU/d) and oral intake without supplementation (212 ± 24 IU/d) among girls aged 9-13 years old was also lower than the RDA (Bailey et al., 2010). From 2007 to 2010, only 1/3 of children reported taking calcium and vitamin D-containing supplements (Bailey et al., 2013; Wallace, Mcburney & Fulgoni, 2014). According to these data, U.S. children need to increase their intakes of calcium and vitamin D.

Other nutrients, such as protein, magnesium, phosphorous, potassium, vitamin C, vitamin K, and zinc are thought to be important for bone growth and maintenance (Laudermilk, et al. 2012; Bergman C, Gray-Scott D, Chen JJ, Meacham S, 2009; Sugiura et al. 2011; Ouzzif et al. 2012). Researchers have found that relatively high dietary protein consumption could promote bone mass accretion during childhood (Bonjour, Chevalley, Rizzoli & Ferrari, 2007), and inconsistent results were reported on the beneficial effect of plant protein over animal protein on bone health (Bonjour, Chevalley, Rizzoli & Ferrari, 2007; Heaney & Layman, 2008). Magnesium has been reported to be positively associated with lumbar spine BMC in healthy Caucasian girls (Carpenter et al., 2006). Phosphorus depletion is thought to cause impaired mineralization (Palacios, 2006). However, little evidence has been found in healthy individuals. More concern is on the impact of high dietary phosphorus on bone, in particular, if combined with a low calcium diet (Palacios, 2006; Takeda, Yamamoto, Yamanaka-Okumura & Taketani, 2012). Nieves et al. (2010) found that a high intake of potassium was associated with significant gains in whole-body BMD and BMC, as well as hip BMD in female adolescents. Severe vitamin C deficiency has been associated with low BMD (Ahmadieh & Arabi, 2011). Due to its role in facilitating osteocalcin synthesis, vitamin K status has been associated with low bone turnover and higher total body and lumbar spine BMC (O'Connor et al., 2010). Zinc is required for osteoblast activity, and studies have associated low serum zinc concentrations and high urinary zinc excretion with osteoporosis (Palacios, 2006). While these nutrients play important roles in bone growth and maintenance, their intakes may be suboptimal, because a recent study has reported that important food group intakes of nearly the entire U.S. population are markedly divergent from dietary recommendations, indicating suboptimal nutrient intakes across the

population (Krebs-Smith et al., 2010). These nutritional deficits could increase the risk of diet-related chronic disease, such as osteoporosis (Krebs-Smith et al., 2010).

In addition to nutrition, physical activity plays a critical role in influencing bone outcomes in terms of timing, type, frequency, and dose (Ishikawa, Kim, Kang & Morgan, 2013; Meyer et. al., 2010). Multiple studies have indicated that bone growth and mineral accrual are most responsive to physical activity and have greater capacity to adapt to mechanical stimulation before puberty or during early puberty (Hind & Burrow, 2007; Bass.,et al. 1998; Ishikawa, Kim, Kang & Morgan, 2013). It is also thought that weight-bearing or impact-loading exercise such as stepping, running, and gymnastic activities result in significantly greater bone accretion compared with non-weight-bearing exercise, such as swimming (Huang et al., 2003; Greene, Naughton, Bradshaw, Moresi & Ducher, 2012). Meyer and colleagues (2010) evaluated the effect of increased school based weight-bearing physical activity through a cluster randomized control trial, reporting a significant increase in total body and lumbar spine BMC and BMD over a nine-month intervention. Higher frequency of physical activity has been positively related to higher BMD. A meta-analysis evaluating the influence of weight-bearing activity on bone health of female children and adolescents, finding that a greater frequency of weight-bearing activities is related to greater lumbar spine BMD. Future studies investigating dose of physical activity and bone outcomes are needed (Ishikawa, Kim, Kang & Morgan, 2013).

Bone strength is determined by both its content and structure (Currey, 2002). Bone mineral content (BMC or bone mass) is an important determinant of bone strength and a useful predictor of fracture risk. Since bone strength cannot be measured in vivo, direct measurements such as

areal bone mineral density (aBMD) and bone mineral content (BMC) are used as alternatives. Children with low BMD and bone mass are thought to be at a higher risk of osteoporosis, which is associated with high risk of fracture (Palacious 2006; Bianchi, 2007). The most common fracture sites are the spine, hip, forearm and proximal humerus (World Health Organization, 2007; Looker, Melton, Harris, Borrud, & Shepherd, 2010). The present study examined lumbar spine bone outcomes in children.

The current gold standard for osteoporosis diagnosis is dual-energy X-ray absorptiometry (DXA), which is traditionally used to provide postero-anterior (PA) measurement of areal BMD (aBMD) and BMC. Areal BMD is thought to be a faulty indicator of volumetric bone density or imperfect indicator of fracture risk, because it does not provide assessment of the spatial distribution of BMD within the bone or bone quality, such as trabecular microstructure (Graeff et. al., 2013). In addition, lumbar spine DXA-derived aBMD fails to distinguish the posterior elements from the vertebral body, which lowers the ability to predict vertebral body strength (Taton et al., 2013). Due to the unsatisfactory predictive ability of aBMD, other bone geometry measurements have been proposed for predicting bone strength, such as volumetric bone mineral density (vBMD) and bone mineral apparent density (BMAD) (Leonard, Shults & Zemel, 2006). However, these measurements are also considered inaccurate for bone strength assessment when without taking three dimensional (3D) bone geometry into account (Cheng et al., 1997). These measurements could be improved by the use of PA DXA scans and supine lateral (LAT) scans, which generate 3 D bone mineral content, density and geometry measurements (Dowthwaite, Rosenbaum, & Scerpella, 2011; Leonard, Shults & Zemel, 2006).

To our knowledge, no studies have utilized this technique to evaluate BMD, BMC or other geometric bone outcomes in relation to dietary intake and dose of physical activity in pre-pubertal girls. In this study, we aim to evaluate how key bone nutrients and dose of physical activity affect bone mass, geometry, and strength in pre-pubertal girls using paired PA and LAT scans to generate three-dimensional DXA assessments. We hypothesize that favorable nutrition and physical activity indices will be positively associated with bone outcomes.

METHODS

Study Design

The data used in this cross-sectional study are from an on-going longitudinal study of bone growth in relation to physical activity of female gymnasts and non-gymnasts (Dowthwaite, Rosenbaum, & Scerpella, 2011). The study was approved by the SUNY Upstate Medical University Institutional Review Board, and is in compliance with US bioethical legislation and the ethical standards of the Declaration of Helsinki. Written parental consent and child assent were obtained from participants.

Participants

Participants in the longitudinal study were recruited from local gymnastics schools, local private schools, and athletic groups. Further details of the participants of the longitudinal study have been published previously (Dowthwaite, & Scerpella, 2011; Scerpella, Davenport, Morganti, Kanaley, & Johnson, 2003). Due to the strong influence of sexual maturity associated hormonal changes on bone mineral mass, using pre-pubertal participants as a study population could help

eliminate confounding factors. Thus only pre-pubertal participants with Tanner breast stage 1 and Tanner pubic stage 1 were included in the data analysis of the present study. One participant was excluded from the data analyses as an outlier due to dietary intakes exceeding 2 standard deviations above the sample mean, particularly for vitamin C and vitamin B12. A total of 50 participants were included in the analyses.

Anthropometry

Height and weight were measured semi-annually. Height was measured via a wall-mounted stadiometer (healthometer) and weight via an electronic digital scale (detecto) (Dowthwaite, Rosenbaum, & Scerpella, 2011). Body Mass Index (BMI) of each participant was calculated (kg/m^2) and BMI-for-age percentiles were plotted using the Centers for Disease Control (CDC) Growth Charts age for Children and adolescents, 2 to 20 years (CDC Growth Chart, Girl 2-20).

Physical Maturity Evaluation

Physical maturity status was recorded semi-annually. Tanner breast and pubic stages (Duke, Litt & Gross, 1980) were used to assess the pubertal status of the subjects. Line drawings of Tanner's stages of development were presented to the participants. In the drawings, five stages of secondary sex characteristics (breast and pubic hair development) were matched to descriptive phrases. Participants were instructed to choose the stage which best represented their own sexual development stage based on their perceptions, with parental assistance as necessary (Dowthwaite, Rosenbaum, & Scerpella, 2012, Dowthwaite, Rosenbaum, & Scerpella, 2011).

Dietary Intake

Participants' usual dietary intakes were evaluated semi-annually using the self-administered semi-quantitative Youth/Adolescent Questionnaire (YAQ, 1995), which was developed for use in children and adolescents by researchers at Harvard University. The questionnaire lists 145 foods and supplements, and standard serving sizes or natural portions of each item (Rockett & Colditz, 1997). The frequency categories range from "never/less than once per month" to "5 or more per day." The response options for frequency of consumption depend on the type of food. The total nutrient score for each item is calculated by multiplying the nutrient content of food by its corresponding frequency score. The validity of the YAQ was evaluated by comparing the average of two YAQs to three 24-hour recalls conducted from 261 youths and adolescents aged 9-18 years. The average energy from theYAQ was higher, but within 1% of the recalls. The means of all nutrients from the two methods differed within 20%, with the exception of vitamin A, carotene, and alcohol (Rockett & Colditz, 1997). Dosage, frequency and length of multivitamin use were taken into account. Dietary intakes without and with supplementation of the focal nutrients were evaluated.

Physical Activity

Total organized, non-aquatic physical activity was recorded semi-annually using a calendar-based form to yield activity-specific participation (hours/week). Based on recruitment for the longitudinal study, girls were grouped as either a gymnast or non-gymnast. The girls in the non-gymnast group were not necessarily sedentary. Hence, instead of focusing on their hours of participation in gymnastics, the current study evaluated participants according to their annual mean organized physical activity levels. To more accurately evaluate the relationship between physical activity dose and bone outcomes, participants' physical activity levels were used as a continuous variable.

Body Composition, Bone Density and Bone Geometry

DXA was used to assess bone in this sample. DXA can rapidly measure large regions of interest, has high precision and accuracy as well as low radiation exposure, and is strongly correlated with fracture risk (Dowthwaite, Flowers & Scerpella, 2011; Mazess, Barden, Bisek & Hanson, 1990; Rizzoli et al., 2010). However, DXA is traditionally used to provide PA measurement of lumbar spine aBMD. Numerous problems have been reported that affect PA DXA lumbar spine assessement, including fan beam magnification error (FBME), failing to account for vertebral depth, and lack of the ability to distinguish the vertebral body BMC (trabecular tissue) from the posterior elements (cortical tissue)(Dowthwaite, Rosenbaum & Scerpella, 2011). Fortunately, DXA skeletal scans are improved by pairing postero-anterior (PA) scans with the supine lateral (LAT) scans (Leonard, Shults & Zemel, 2006). In addition to reducing the confounding effects of FBME, LAT scans can isolate BMC from the vertebral body only, which eliminates the posterior element. Paired DXA scans also provide a three-dimension lumbar spine assessment including vertebral width (PAWIDTH), depth (LATDEPTH), and height (LATVHEIGHT). These assessments yield more accurate bone geometry and total bone mineral apparent density (BMAD) than those derived from 2 dimensional PA or LAT scans alone. This allows superior estimation of three-dimensional bone structure and strength indices (Dowthwaite, Rosenbaum, & Scerpella, 2011).

At the annual DXA measurements, body composition, including total body non-bone lean mass, lean mass, and fat mass were measured by total body DXA scans. PA and LAT lumbar spine DXA scans were performed to obtain poster-anterior bone mineral density (PABMD), poster-anterior bone mineral content (PABMC), poster-anterior width (PAWIDTH), supine lateral height (LATVHEIGHT) and supine lateral bone mineral content (LATBMC). All scans were

analyzed by a single investigator (JD) using Apex software (Hologic Discovery A, software v.12.7.3, Waltham, MA, USA).

Only data from lumbar vertebra 3 (L3) were included in the analyses as L2 and L4 are often overlapped by rib and pelvic bone, respectively, when included in the region of interest in lateral scans (Rupich, Griffen, Pacifici, Avioli & Susman, 1992; Dowthwaite, Rosenbaum, & Scerpella, 2011). Plane-specific bone geometric indices were calculated, including mean vertebral width (PAWIDTH=PAAREA/LATVHEIGHT) and mean vertebral depth (LATDEPTH= LATAREA/LATVHEGHT). Vertebral volume (PALATV), volumetric density (PALATBMAD), structural strength in axial compression (IBS), and fracture risk index (FRI) were calculated using formulae based on simplified geometric models (Dowthwaite, Rosenbaum, & Scerpella, 2011). Instead of Hologic standard-width-adjusted BMD and width-adjusted volume, investigator-calculated PALATV and PALATBMAD were used for data analysis. These calculations were performed, because the standard Hologic output erroneously uses only lateral bone geometric indices, whereas we incorporate PA bone width to assess 3D geometry. Detailed descriptions of these calculations have been published previously (Dowthwaite, Rosenbaum, & Scerpella, 2011).

Data Analysis

Descriptive statistics (means, SDs, and ranges) were computed. Data from dependent (bone) variables were normally distributed with the exception of bone fracture risk index. Thus, bone fracture risk index was converted to natural logarithms for analysis and back-transformed for presentation of results. Pearson correlation coefficients were calculated to evaluate the bivariate

relationships between dietary intakes and bone measurements. Multiple linear regressions were used to examine the associations between key dietary variables, physical activity and bone measurements in all subjects, with adjustments for age, height, and annual mean physical activity. The alpha level was set at $p<0.05$ for all tests. All data analyses were performed using SPSS for Windows statistical software, version 21 (SPSS, Inc., Chicago, IL).

RESULTS

Participant Characteristics

Participants included in the analyses were 7 to12 years old. Descriptive characteristics of the subjects are shown in Table 1. The mean percentile rank for BMI for age was 43.08 (SD=25.34, 4-98). The means for non-bone lean body mass and fat mass were 19.63kg (SD=3.74, range 13.25-31.68) and 5.80 kg (SD=2.41, 1.89-16.78) respectively.

Dietary Intake

Daily mean intakes of energy, macronutrients, and micronutrients are shown in Table 2. The mean intakes of the focal nutrients that met the RDA were as follows: carbohydrate, protein, phosphorus, magnesium, vitamin A, vitamin C, zinc, and vitamin B12. The mean intakes of fiber, calcium, vitamin D, and potassium were below the RDA. The biggest nutrient of concern was potassium since only 8% of the participants met the RDA for this nutrient. For all other nutrients of interest excluding potassium, 70% of subjects met the EAR with supplementation; 18% met the EAR without supplementation. Fifty percent of the subjects exceeded the RDA when

including supplements; whereas only 2% of the subjects met or exceeded the RDA without supplementation.

Dosage and length of multivitamin/mineral (MVMM) intakes are described in Table 3. Fifty-nine percent of participants reported taking MVMM supplements.

Physical Activity

On average, the annual mean non-aquatic organized activity exposure was 6.9 ± 5.1 hours per week. In total, 30% of subjects met the physical activity recommendations of ≥ 1 h/day (on average, approximated as at least 7 h/wk). Physical activity levels were variable and ranged from 0 to 21.5 hours per week.

Bone Outcomes

PABMC, PABMD, LATHEIGHT, and LATWIDTH were significantly correlated with carbohydrate and fiber intakes (Table 4). No significant correlations were found for intakes of vitamin D and calcium and the bone measurements. The relationships between the focal nutrients and bone outcomes after adjustment for age, height, and physical activity level are shown in Table 5.

Physical activity was positively associated with PABMD, IBS, and FRI (Table 6). Significant associations were also found between physical activity and PABMD, PABMC, PAWIDTH, LATBMC, PALATIBS, and FRI after adjusting for age, height, and all the key nutrients (Table 6).

DISCUSSION

To our knowledge, this is the first study to evaluate the relationship between diet, physical activity, and bone outcomes as measured by paired three-dimensional DXA scans in pre-pubertal girls. We found that carbohydrate, fiber, and zinc intakes were correlated with PABMC, PABMD, LATHEIGHT, and LATWIDTH. However, with the exception of carbohydrate intake and LATHEIGHT, the associations were no longer significant after adjusting for age, height, and level of physical activity. We observed a significant negative association between carbohydrate intake and LATHEIGHT, which has not been previously reported in the literature.

The lack of significant associations between diet and bone outcomes may be a result of adequate energy and nutrient intakes of the participants. The participants in this study met the dietary reference intakes (DRI) for energy and all the macronutrients, as well as most of the key bone nutrients even without taking their dietary supplement into account. In addition to their optimal oral intake, 59% of the participants also reported taking MVMM supplements, which is higher than the average intake of dietary supplements (31%) in children in the United States (Dwyer et al., 2013; Bailey et al., 2013).

A recent study investigating the motivation for use of dietary supplements among children reported that the primary reasons for supplement use was to improve or maintain health (Bailey et al., 2013). The use of dietary supplementation has been found to be higher among individuals reporting excellent or very good health (Bailey et al., 2013). Weight status and screen time were found to be inversely related to supplement use while physical activity level and healthy dietary habits were positively associated with supplement use. Additionally, high intakes have been reported in children of non-Hispanic white descent, with higher family income, and with those

who have health insurance (Bailey et al., 2013). Although we did not assess socio-economic status directly, the majority of our participants were Caucasian and on average reported "adequate" dietary intake. On that basis, our results appear to corroborate overall patterns observed by Bailey et al (2013) in white, non-Hispanic, middle to upper SES children.

For calcium and vitamin D, although the mean intakes for our subjects were slightly below the recommended dietary allowance (RDA), they did meet the estimated average requirement (EAR) of Calcium and vitamin D (Vitamin D, fact sheet for health professionals). Although we did not take into account seasonal influence, the RDA is based on an assumption of minimal sun exposure. Therefore it is a legitimate assumption that most of our participants had adequate dietary calcium and vitamin D intakes. Our study did not detect an association between calcium or vitamin D and bone outcomes, while past studies reported high dietary or serum calcium and vitamin D being associated with high bone mass accrual among pre-pubertal children. However, in those studies, the average intakes of these nutrients were below recommendations and sunlight exposure was likely to be low based on the latitude where the population resided (Finland) (Cheng, et al., 2003; Cheng, et al., 2005). Discordance in dietary adequacy coupled with differences in sunlight exposure could explain discrepancies between studies, as the positive relationship between key bone nutrients and bone outcomes may not be as pronounced in well-nourished children compared to those with nutrient deficits.

Our analyses detected positive associations between physical activity exposure and multiple bone outcomes including PABMC, PABMD, PAWIDTH, PALATIBS, and FRI. These associations were significant after adjusting for age, height and each key bone nutrient. Overall, our results support the importance of physical activity to lumbar vertebral mineral density and content, vertebral width, and vertebral strength. Only 30% of the participants' average weekly

hours of physical activity met the recommendation (60 minute/day) (Physical activity guidelines, 2008), and the coefficient of variation for physical activity is high (CV=74%), which indicates high activity variability. Our results may indicate that physical activity plays a stronger role in bone outcomes than diet. Alternatively, it is easier to detect the effect of physical activity when the majority of the participants did not meet the physical activity recommendation based on their reported organized physical activity. It may also be possible that in our subjects, failure to detect dietary associations may be due to inadequate variability in dietary intakes, in contrast to highly variable physical activity.

Similar to our findings, a large body of studies have reported a positive relationship between loading exposure and PA vertebral aBMD and BMC in pre-pubertal girls (Meyer et al., 2010; Valdimarsson et al., 2005; Laing et al., 2005; Tobias, et al., 2006; Linden et al., 2006; Macdonald et al., 2008; Lehtonen-Veromaa et al., 2000). In addition, we observed a significant relationship between physical activity and LATBMC. LATBMC is thought to be superior to PABMC, because lateral DXA scans allow isolation of the vertebral body (main osteoporotic fracture site) from the posterior elements (Dowthwaite, Rosenbaum, & Scerpella, 2011). Our lateral scan BMC results confirmed positive associations between L3 bone mass and loading exposure detected in our previous work evaluating gymnastic training history as an osteogenic factor in subjects of heterogeneous physical maturity (Dowthwaite, Rosenbaum, & Scerpella, 2011). Notably, the present study was able to detect significant positive associations between **dose** of physical activity and L3 bone mass and density, **after** accounting for the statistical effects of multiple key nutrient intakes; this finding has not been previously reported.

In addition to bone mass and density, bone geometry is also an important determining factor for bone strength and fracture risk. Bone geometry influences bone strength independent of bone

mass (Duan, Parfitt & Seeman, 1999; Ahlborg et al., 2003). However, in past studies, bone geometry was often considered a confounding factor rather than an outcome measurement that may be affected by nutrition and physical activity (Esterie et al., 2010; Tobias, et al., 2006; Lehtonen-Veromaa et al., 2000; Grimston, Willows & Hanley, 1993). Evaluating the relationship between L3 width, height, depth and physical activity, our results for PAWIDTH show a significant positive association between L3 width and physical activity exposure. The results are consistent with findings reported by a 1-year school-based exercise intervention study with girls at Tanner stage I (Valdimarsson et al., 2006), and a 3-year exercise intervention program with pre-pubertal children (Lofgren et al., 2011).

In contrast, we did not detect significant associations between LATDEPTH, LATHEIGHT, and physical activity. In contrast to the current analysis, our prior work reported lower LATHEIGHT and wider PAWIDTH among pre- and post-menarcheal females exposed to at least 6 h/wk of gymnastic activity, compared to females with little or no gymnastic exposure, accounting for variability in age, maturity and body size. It is possible that additional variability conferred by the inclusion of pubertal and post-menarcheal subjects empowered detection of loading associations with vertebral width and height. In that analysis, it was postulated that LATDEPTH expansion in relation to loading may be diminished by tensile resistance from posterior elements and ligamentous connections, while the enlargement of vertebral PAWIDTH might be required due to the relative absence of medial-lateral accessory support against loading (Dowthwaite, Rosenbaum, & Scerpella, 2011).

It is thought that pairing of PA DXA scans with LAT scans may increase sensitivity to measure trabecular BMC and density variability (Leonard, Shults & Zemel, 2006; Dowthwaite, Rosenbaum, & Scerpella, 2011). Nevertheless, in the present study, no significant association

was found between PALATBMAD, PALATV, and physical activity. PALATV was significantly associated with height of participants (data not shown), yielding a very low adjusted model R square for both linear and quadratic regression (see Table 5). PALATBMAD was not associated with any of the independent variables, including age, height, physical activity, or nutrient intake. It is possible that our calculation of BMAD is not an accurate assessment of volumetric BMD. Alternatively, BMAD (or vBMD) may not be responsive to focal dietary variables or loading exposures. Overall, our lack of associations between loading and PALATBMAD and PALATV are similar to analyses of pre- and post-menarcheal Ex/Gymnasts vs Non-gymnasts without evaluating dietary variables or loading dose (Dowthwaite, Rosenbaum, & Scerpella, 2011). It is possible that BMAD (and vBMD) are primarily determined by genetic factors.

We identified significant positive associations between calculated PALATIBS and physical activity levels, and negative associations between calculated FRI and physical activity. In contrast with studies that reported higher fracture risk in children with higher physical activity (Lofgren et al., 2013), we did not evaluate actual fracture incidence or consider exposure to trauma as an influential factor.

This study has several strengths. The participants were a homogeneous group based on race and geographical location, which helps to minimize confounding factors. In particular, the pre-pubertal status of all the participants avoided the influence of variable hormonal exposure during and after puberty. Furthermore, the current study investigated associations of dietary intake versus lumbar spine bone mass, geometry, and strength, while accounting for physical activity. When evaluating the association between physical activity dose and lumbar spine bone outcomes, the statistical effect of each focal nutrient was taken into account. Therefore the findings reflect diet and physical activity as factors in bone outcomes in well-nourished children, allowing

comparison of diet and physical activity explanatory value. The paired 3D provided us more accurate prediction for bone mineral content, density, geometry, and strength. Use of the YAQ questionnaire allowed us to assess the intakes of each focal nutrient through diet and supplementation. This detail has only been evaluated by a few past studies investigating the relationship between dietary intake and bone outcomes (Merrilees et al., 2000; Laudermilk et al., 2012). The assessment of both dietary and supplement intake provided us the advantage of comparing the influence of nutrients from different sources on bone outcomes in children.

This study is not without limitations. First and foremost, the study did not take genetics into account, and genetics accounts for 60-80% of the variation of peak bone mass. Also, due to its cross-sectional design, we were unable to measure change in participants' bone mass over time. Future longitudinal analyses will provide information on the influence of key bone nutrients and physical activity dose on bone growth. In addition, due to the secondary nature of this analysis, the participants were not recruited based on nutrition-related objectives. Furthermore, the exclusion of pubertal and mature subjects from the parent study led to a relatively small sample size, which reduced statistical power.

The participants of this study are a relatively homogeneous group based on race, ethnicity and presumed socioeconomic status. They appear to be guided and supported to develop healthy eating habits and participate in extracurricular physical activities. Associations between nutrient intake and bone outcomes in well-nourished children might not be as obvious as in children with variable levels of nutrient insufficiency. Furthermore, although the average aBMD and BMC of the participants were less than CDC references (CDC 2005-2008), they may actually have higher L3 bone mass and density compared to girls of the same body size (See Table 7 and Table 8). The participants' short stature and lighter body weight (Table 6) could be a result of later onset of

puberty. We did not have all of the menarche dates for our participants. It is possible that girls in the study had later onset of puberty compared to the CDC population with the same race (Menstruation in Girls and Adolescents, 2006).

The subjects of the parent longitudinal study were recruited based on their age and level of participation in gymnastics activity. Studies have reported that different types of physical activity have different effects on bone mineral acquisition. Weight-bearing or impact-loading exercise such as stepping, running, and gymnastics in particular, results in significantly greater bone accretion compared with non-weight-bearing exercise, such as swimming (Huang et al., 2003; Greene et al., 2012). A prior analysis by our group reported a significant difference in lumbar spine bone outcomes between pre- and post-menarcheal gymnasts and non-gymnasts after adjusting for age, height, and physical maturity (Dowthwaite, Rosenbaum, & Scerpella, 2011). It may be that gymnastic exposure is the primary driver of the observed activity dose statistical effects.

Although the participants of the current study were all pre-pubertal, hormonal levels could still be an important influencing factor for bone growth, However, due to unknown menarche dates for most of the subjects, chronological age was used in analyses instead gynecological age; the latter is a better indicator of maturity and associated hormonal status.

Due to the large number of nutritional predictors and bone outcomes evaluated, it is possible that the few significant correlations could be attributed to chance alone (Type I error). By using an alpha level of 0.05, 1.6 significant associations with bone variables would be expected due to

random chance. On this basis, our significant nutritional associations should be viewed with caution.

CONCLUSION

We did not identify significant associations between total dietary intake or diet and supplement intake and bone outcomes in this cohort of well-nourished and highly active pre-pubertal girls. Physical activity appears to play a stronger role in influencing bone outcomes than nutrient intakes. Higher level of physical activity was significantly associated with higher third lumbar vertebral bone mass, areal density, vertebral width and theoretical bone strength in axial compression, as well as lower risk fracture index. We did not observe significant relationships between physical activity and vertebral height, depth, paired volumetric bone density or bone volume. Future studies investigating the influence of genetics on bone outcomes are warranted. Furthermore, studies with a more diverse cohort composition in terms of race, ethnicity, nutrient intake, and socio-economic status are also recommended.

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4. List of Illustrative Materials

Table 1. Descriptive characteristics of pre-pubertal girls, with Tanner breast stage 1 and Tanner pubic stage 1. (n=50)		
	Mean ± SD	**Range**
Age	9.5 ± 1.2	7.8-12.8
Weight (kg)	29.2 ± 5.4	19.8-45.2
Height (standing, cm)	133.1 ± 8.8	116.0-156.5
BMI (ht in kg/wt in m^2)	16.4 ± 1.8	13.5-24.8
BMI for age percentile	43.1± 25.3	4-98
Fat Mass (kg)	5.8 ± 2.4	1.9-16.8
Total Lean Mass (kg)	20.4 ± 3.9	13.8 -33.0
Soft lean tissue Mass (kg)	19.6 ± 3.7	13.3-31.7
Total Lean Mass (%)	78.4 ± 5.0	59.1- 88.7
Fat Mass (%)	21.7 ± 5.0	11.3 - 40.9
Physical Activity Level (h/wk)	6.9 ± 5.1	0.1-21.5
PABMC (g)	5.25 ± 1.28	3.32-9.58
PABMD (g/cm^2)	0.623 ± .073	0.461-0.883
PAWIDTH (cm)	5.69 ± 0.45	4.66-7.20
LATBMC (g)	3.22 ± 0.76	1.95-5.73
LATHEIGHT (cm)	1.47 ± 0.20	1.08-1.93
LATDEPTH (cm)	3.72 ± 0.25	3.07-4.43
PALATV (cm^3)	24.46 ± 4.65	15.53-37.81
PALATBMAD (g/cm^3)	0.13 ± 0.01	0.10-0.15
PALATIBS (g^2/cm^4)	0.29 ± 0.07	0.17-0.58
FRI	1.66E-04 ± 1.73E-06	1.01E-04-2.82E-04

PA= Postero-anterior; BMAD= bone mineral apparent density; BMC= bone mineral content; LAT= lateral; PAWIDTH= postero-anterior vertebral width; LATVHT= lateral vertebral height (roughly equivalent to PA vertebral height); LATDEPTH= lateral vertebral depth; IBS= index of structural strength in axial compression; BMD= areal BMD; PALATV= paired postero-anterior and lateral volume; PALATBMAD= paired postero-anterior and lateral bone mineral apparent density. FRI=Fracture risk index.

Table 2. Dietary intakes and dietary recommendations of 9-13 year old girls (n=50)			
	Total Sample Mean ± SD	**EAR**	**RDA/AI**
Energy (kcal/d)	2137.26 ± 571.20		1800-2200
Protein **(g/d)** **(g/kg/d)**	 88.26 ± 28.48 g/d 3.82 ± 0.90 g/kg/d	 0.76g/kg/d	 34g/d
Protein (percentage of kcal)	19.56 ± 0.50%		10-30%
Total fat (g/d)	71.74 ± 22.53		
Total fat (percentage of kcal)	31.76 ± 2.46%		25-35%
Carbohydrate (g/d)	292.24 ± 76.72	100	130
Carbohydrate (percentage of kcal)	49.43 ± 3.53%		45-65%
Fiber (AOAC) (g/d)	18.64 ± 5.21		26
Calcium (mg/d)	1289.36 ± 534.54	1,100	1,300
Calcium wo (mg/d)	1224.42 ± 530.76		
Vitamin D (IU/d)	530.60 ± 332.21	400	600
Vitamin D wo (IU/d)	270.84 ± 166.79		
Phosphorus (mg/d)	1603.52 ± 516.99		1250
Phosphorus wo (mg/d)	1538.58 ± 511.82		
Potassium (mg/d)	2945.81 ± 921.43		4500
Potassium wo (mg/d)	2945.81 ± 921.43		
Magnesium (mg/d)	304.93 ± 82.60		240
Magnesium wo (mg/d0	291.94 ± 80.96		
Vitamin C (mg/d)	147.15 ± 70.05		45
Vitamin C wo (mg/d)	112.19 ± 54.30		
Zinc (mg/d)	22.06 ± 11.46	7	8
Zinc wo (mg/d)	12.32 ± 3.60		
Vitamin A (RAE/d)	1848.887 ±1185.472		600
Vitamin A wo (RAE/d)	878.89 ± 318.71		

Wo: oral intake without supplementation.

Table 3. Multivitamin Supplement Intake Dosage and Length.

	Taking MVMM supplements	**Not taking supplements**
Number of Participants	29	21
Percentage of Participants	58%	42%

Number of MVMM pills taken per week	0	1	2	3	4
Number of participants	21	3	7	10	9
Percentage of participants	42%	6%	14%	20%	18%

Number of years of taking MVMM supplements	0	1	2	3	4	5
Number of participants	21	5	12	10	1	1
Percentage of participants	42%	10%	24%	20%	2%	2%

Table 4 Associations between physical activity, nutrients, and bone outcomes.

	Physical activity	Carbohydrate	Fiber	Vitamin D	Vitamin D wo	Calcium	Calcium wo
PABMC	0.14	-0.03	**-0.29***	-0.09	0.11	0.11	0.132
PABMD	**0.258***	0.116	**-0.238***	-0.119	0.262	0.117	0.142
PAWidth	0.221	0.121	-0.109	-0.083	0.070	0.115	0.134
LATHEIGHT	-0.129	**-0.281***	**-0.278***	-0.007	0.026	-0.031	-0.026
LATDEPTH	0.057	0.088	**-0.258***	-0.039	0.137	0.172	0.194
LATBMC	0.109	-0.060	-0.258	-0.071	0.127	0.115	0.138
PALATV	0.039	-0.091	-0.250	-0.057	0.197	0.112	0.134
PALATBMAD	0.173	-0.005	-0.171	-0.066	0.058	0.036	0.049
PALATIBS	**0.269***	0.091	-0.182	-0.084	0.123	0.146	0.170
LNFRI	**-0.279***	-0.106	0.005	0.199	-0.024	-0.017	-0.049

Pearson correlation coefficients.

Bold with asterisk: *p<0.05

Wo: without supplement

Table 5 Multiple Linear Regressions. Key nutrients and bone outcomes. (Each model includes: age, height, physical activity, and nutrient of interest)								
	Carbohydrate		**Fiber**		**Vitamin D**		**Calcium**	
	Adjusted R^2	**Beta**	**Adjusted R^2**	**Beta**	**Adjusted R^2**	**Beta**	**Adjusted R^2**	**Beta**
		95% Confidence Interval		**95% Confidence Interval**		**95% Confidence Interval**		**95% Confidence Interval**
PABMC	0.545	-0.027	0.586	-0.196	0.596	-0.125	0.545	0.009
		(-0.004, 0.003)		(-0.096, -0.002)		(-0.001, 0)		(0, 0.001)
PABMD	0.273	0.079	0.312	-0.206	0.282	-0.124	0.273	0.077
		(0, 0)		(-0.006,0)		(0, 0)		(0, 0)
PAWIDTH	0.200	0.069	0.266	-0.074	0.202	-0.085	0.199	0.065
		(-0.001, 0.002)		(-0.029, 0.016)		(0, 0)		(0, 0)
LATHEIGHT	**0.551**	**-0.214***	0.535	-0.167	0.508	-0.060	0.526	-0.147
		(-0.001, 0)		(-0.014, 0.001)		(0, 0)		(0, 0)
LATDEPTH	0.209	0.078	0.204	0.027	0.206	-0.059	0.276	0.480
		(-0.001,0.001)		(-0.011, 0.014)		(0, 0)		(0, 0)
LATBMC	0.569	-0.044	0.593	-0.157	0.576	-0.095	0.567	0.020
		(-0.002, 0.002)		(-0.051, 0.004)		(-0.001, 0)		(0, 0)
PALATV	0.664	-0.070	0.677	-0.132	0.668	-0.098	0.659	-0.011
		(-0.015, 0.006)		(-0.267, 0.030)		(-0.004, 0.001)		(-0.002, 0.001)
PALATBMAD	0.003	0.002	0.030	-0.160	0.005	-0.046	0.005	0.053
		(0, 0)		(-0.001, 0)		(0, 0)		(0, 0)
PALATIBS	0.278	0.072	0.292	-0.134	0.227	-0.066	0.287	0.121
		(0, 0)		(-0.005, 0.001)		(0, 0)		(0, 0)
LNFRI	0.187	-0.039	0.188	0.046	0.203	0.134	0.193	-0.088
		(10.001, 0.001)		(-0.009, 0.013)		(0, 0)		(0, 0)

Pearson correlation coefficients;

Bold with asterisk: *p<0.05

Table 6 Multiple Linear Regressions. Physical activity and bone outcomes. (Each model includes: age, height, physical activity, and nutrient of interest)

	PABMC		PABMD		PAWIDTH		LATHEIGHT		LATDEPTH		LATBMC		PALATBMAD		PALATV		PALATIBS		FRI LN	
	R	**Beta**	**R**	**Beta**	**R**	**Beta**	**R**	**Beta**	**R**	**Beta**	**R**	**Beta**		**Beta**		**Beta**		**Beta**		**Beta**
		95 CI		**95 CI**		**95 CI**		**95 CI**		**95 CI**		**95 CI**	R	**95 CI**	R	**95 CI**	**R**	**95 CI**	**R**	**95 CI**
		0.305*		**0.450****		**0.438****		-0.154		0.222		**0.244***		0.181		0.177		**0.423**		**-0.355***
Energy	**0.545**	**(0.018, 0.136)**	**0.273**	**(0.002,0.011)**	**0.262**	**(0.011, 0.066)**	0.534	(-0.015, 0.003)	0.210	(-0.004,0.025)	**0.567**	**(0.002-0.071)**	0.003	(0, 0.001)	0.661	(-0.024, 0.343)	**0.278**	**(0.002, 0.009)**	**0.254**	**(-0.029, -0.002)**
		0.305*		**0.459****		**0.443****		-0.174		0.235		**0.244***		0.181		0.174		**0.432**		**-0.362***
Protein	**0.545**	**(0.018,0.136)**	**0.269**	**(0.002, 0.011)**	**0.196**	**(0.012, 0.067)**	0.520	(-0.016,0.003)	0.216	(-0.003, 0.026)	**0.602**	**(0.002-0.071)**	0.003	(0, 0.001)	0.659	(-0.027, 0.341)	**0.277**	**(0.002, 0.009)**	**0.188**	**(-0.029, -0.002)**
		0.310*		**0.440****		**0.427****		-0.125		0.213		**0.252***		0.181		0.174		**0.432**		**-0.351***
Carbohydrate	**0.583**	**(0.018, 0.138)**	**0.273**	**(0.002, 0.010)**	**0.200**	**(0.010, 0.066)**	0.551	(-0.014, 0.003)	0.213	(-0.005, 0.025)	**0.569**	**(0.002-0.071)**	0.004	(0, 0.001)	0.664	(-0.027, 0.341)	**0.278**	**(0.002, 0.009)**	**0.187**	**(-0.029, -0.002)**
		0.305*		**0.458****		**0.440****		-0.169		0.230		**0.243***		0.181		0.188		**0.429**		**-0.362***
Total fat	**0.545**	**(0.018,0.136)**	**0.272**	**(0.002, 0.011)**	**0.262**	**(0.011, 0.066)**	0.512	(-0.016, 0.003)	0.205	(-0.004, 0.026)	**0.567**	**(0.002-0.070)**	0.003	(0, 0.001)	0.659	(-0.027, 0.341)	**0.275**	**(0.002, 0.009)**	**0.188**	**(-0.029, -0.002)**
		0.313*		**0.464****		**0.444****		-0.159		0.227		**0.249***		0.188		0.179		**0.432**		**-0.361***
Fiber	**0.586**	**(0.022, 0.135)**	**0.312**	**(0.002, 0.011)**	**0.201**	**(0.012, 0.067)**	0.535	(-0.016, 0.003)	0.204	(-0.004,0.026)	**0.593**	**(0.004-0.071)**	0.030	(0, 0.001)	0.677	(-0.017, 0.341)	**0.292**	**(0.002, 0.009)**	**0.188**	**(-0.029, -0.002)**
		0.206*		**0.462****		**0.446****		-0.179		0.236		**0.245***		0.186		0.173		**0.432**		**-0.366***
Calcium	**0.582**	**(0.018, 0.136)**	**0.273**	**(0.002, 0.011)**	**0.199**	**(0.012, 0.067)**	0.565	(-0.016, 0.003)	0.212	(-0.004,0.026)	**0.567**	**(0.002-0.071)**	0.005	(0, 0.001)	0.659	(-0.027, 0.341)	**0.287**	**(0.002, 0.010)**	**0.193**	**(-0.029, -0.002)**
		0.307*		**0.462****		**0.446****		-0.176		0.235		**0.246***		0.186		0.174		**0.432**		**-0.362***
Calcium wo	**0.546**	**(0.018, 0.136)**	**0.277**	**(0.002, 0.011)**	**0.201**	**(0.012, 0.067)**	0.525	(-0.016, 0.002)	0.214	(-0.004,0.026)	**0.568**	**(0.002-0.071)**	0.007	(0, 0.001)	0.659	(-0.027, 0.341)	**0.292**	**(0.002, 0.009)**	**0.199**	**(-0.029, -0.002)**
		0.282*		**0.433****		**0.425****		-0.177		0.217		**0.226***		0.173		0.156		**0.437**		**-0.334***
Vitamin D	**0.560**	**(0.012, 0.129)**	**0.282**	**(0.002, 0.010)**	**0.202**	**(0.012, 0.067)**	0.508	(-0.016, 0.003)	0.206	(-0.004,0.026)	**0.610**	**(-0.001-0.068)**	0.005	(0, 0.001)	0.668	(-0.042, 0.324)	**0.277**	**(0.002, 0.009)**	**0.203**	**(-0.029, -0.002)**
		0.309*		**0.463****		**0.444****		-0.174		0.235		**0.249***		0.191		0.174		**0.440**		**-0.369***
Vitamin D wo	**0.546**	**(0.018, 0.137)**	**0.272**	**(0.002, 0.011)**	**0.196**	**(0.012, 0.067)**	0.510	(-0.016, 0.003)	0.207	(-0.004,0.026)	**0.569**	**(0.003-0.071)**	0.010	(0, 0.001)	0.659	(-0.027, 0.341)	**0.285**	**(0.002, 0.009)**	**0.194**	**(-0.029, -0.002)**

Bold with double asterisk: **P<0.01

R: Adjusted R square

B: Beta

CI: 95 Confidence interval

wo: oral intake without supplement

Table 6 Continue

	PABMC		PABMD		PAWIDTH		LATHEIGHT		LATDEPTH		LATBMC		PALATBMAD		PALATV		PALATIBS		FRI LN	
Phosphorous	**0.545**	**0.303* (0.017, 0.135)**	**0.269**	**0.460** (0.002, 0.011)**	**0.196**	**0.444** (0.012, 0.067)**	0.525	-0.181 (-0.016, 0.002)	0.210	0.237 (-0.004,0.026)	**0.567**	**0.243* (0.002-0.071)**	0.003	0.184 (0, 0.001)	0.660	0.171 (-0.030, 0.339)	**0.280**	**0.436 (0.002, 0.010)**	**0.189**	**-0.365* (-0.029, -0.002)**
Phosphorous wo	**0.545**	**0.306* (0.018, 0.136)**	**0.271**	**0.461** (0.002, 0.011)**	**0.197**	**0.445** (0.012, 0.067)**	0.524	-0.178 (-0.016, 0.003)	0.213	0.237 (-0.003,0.026)	**0.567**	**0.245* (0.002-0.071)**	0.004	0.185 (0, 0.001)	0.687	0.173 (-0.027, 0.341)	**0.284**	**0.468 (0.002, 0.009)**	**0.192**	**-0.366* (-0.029, -0.002)**
Potassium	**0.548**	**0.304* (0.017,0.135)**	**0.267**	**0.456** (0.002, 0.011)**	**0.195**	**0.440** (0.012, 0.067)**	0.529	-0.170 (-0.016, 0.003)	0.269	0.229 (-0.004,0.026)	**0.568**	**0.242* (0.002-0.070)**	0.004	0.182 (0, 0.001)	0.665	0.172 (-0.026, 0.338)	**0.275**	**0.429 (0.002, 0.009)**	**0.185**	**-0.359* (-0.029, -0.002)**
Potassium wo	**0.548**	**0.304* (0.017, 0.135)**	**0.267**	**0.456** (0.002, 0.011)**	**0.195**	**0.440** (0.012, 0.067)**	0.529	-0.170 (-0.016, 0.003)	0.204	0.229 (-0.004,0.026)	**0.568**	**0.242* (0.002-0.071)**	0.004	0.182 (0, 0.001)	0.665	0.172 (-0.067, 0.338)	**0.272**	**0.432 (0.002, 0.009)**	**0.185**	**-0.359* (-0.029, -0.002)**
Magnesium	**0.554**	**0.303* (0.018, 0.134)**	**0.271**	**0.454** (0.002, 0.011)**	**0.198**	**0.439** (0.012, 0.067)**	0.523	-0.169 (-0.016, 0.003)	0.203	0.228 (-0.004,0.026)	**0.571**	**0.242* (0.002-0.070)**	0.003	0.181 (0, 0.001)	0.667	0.172 (-0.026, 0.337)	**0.273**	**0.427 (0.002, 0.009)**	**0.185**	**-0.359* (-0.029, -0.002)**
Magnesium wo	**0.549**	**0.305* (0.018, 0.135)**	**0.268**	**0.456** (0.002, 0.011)**	**0.196**	**0.441** (0.011, 0.067)**	0.522	-0.166 (-0.016, 0.003)	0.269	0.228 (-0.004,0.026)	**0.569**	**0.243* (0.002-0.071)**	0.003	0.181 (0, 0.001)	0.691	0.174 (-0.025, 0.340)	**0.273**	**0.427 (0.002, 0.009)**	**0.186**	**-0.359* (-0.029, -0.002)**
Vitamin C	**0.577**	**0.340* (0.028, 0.143)**	**0.292**	**0.487** (0.003, 0.011)**	**0.217**	**0.470** (0.014, 0.069)**	0.506	-0.158 (-0.016, 0.003)	0.235	0.263 (-0.002,0.027)	**0.597**	**0.277* (0.008-0.075)**	0.005	0.190 (0, 0.001)	0.692	0.210 (-0.013, 0.367)	**0.299**	**0.459 (0.002, 0.010)**	**0.256**	**-0.411* (-0.029, -0.002)**
Vitamin C wo	**0.593**	**0.348* (0.026, 0.149)**	**0.272**	**0.482** (0.002, 0.011)**	**0.207**	**0.483** (0.012, 0.067)**	0.506	-0.152 (-0.016, 0.004)	0.229	0.291 (-0.002,0.029)	**0.617**	**0.292* (0.008-0.079)**	0.003	0.172 (0, 0.001)	0.681	0.232 (-0.023, 0.397)	**0.277**	**0.432 (0.002, 0.009)**	**0.224**	**-0.435* (-0.029, -0.002)**
ZInc	**0.571**	**0.276* (0.012, 0.127)**	**0.299**	**0.424** (0.002, 0.010)**	**0.210**	**0.419** (0.009, 0.065)**	0.507	-0.174 (-0.016, 0.003)	0.209	0.214 (-0.005,0.025)	**0.583**	**0.221* (-0.001-0.067)**	0.011	0.166 (0, 0.001)	0.673	0.153 (-0.043, 0.320)	**0.287**	**0.407 (0.001, 0.009)**	**0.231**	**-0.322* (-0.029, -0.002)**
Zinc wo	**0.545**	**0.303* (0.017, 0.135)**	**0.267**	**0.454** (0.002, 0.011)**	**0.196**	**0.436** (0.012, 0.067)**	0.509	-0.174 (-0.016, 0.003)	0.206	0.234 (-0.004,0.026)	**0.567**	**0.244* (0.002-0.071)**	0.003	0.184 (0, 0.001)	0.687	0.173 (-0.027, 0.341)	**0.274**	**0.432 (0.002, 0.009)**	**0.188**	**-0.353* (-0.029, -0.002)**

Bold with double asterisk: **P<0.01

R: Adjusted R square

B: Beta

CI: 95 Confidence interval

wo: oral intake without supplement

Table 7: Comparison between mean L3 vertebral bone area, content, and density of study participants and NHANES references			
	Mean of participants	**NHANES references**	**Percentage of particpants' mean bone outcome compared to the reference**
L3 PA AREA	8.35±1.21	10.48±1.46	**<5th**
L3 PABMC	5.26±1.30	7.23±2.05	**10th-15th**
LA PABMD	0.62±0.07	0.68±0.11	**25th-50th**

Bold numbers: standard weight and height referenced from CDC website.

http://www.cdc.gov/nchs/data/series/sr_11/sr11_251.pdf.

Table 8: Comparison between mean height and weight of study participants and the CDC references				
	Mean of participants		**CDC references**	
	Weight	**Height**	**Weight**	**Height**
7 yo (n=1)	45.2 (>90th)	135.0 (>90th)	**27.3±0.62**	**125.8±0.8**
8 yo (n=20)	26.1 (25th-50th)	127.9 (25th-50th)	**30.7±0.94**	**131.3±0.5**
9 yo (n=14)	29.6 (25th)	132.4 (15th-25th)	**36.7±0.99**	**138.6±0.7**
10 yo (n=11)	30.8 (15th-25th)	139.0 (25th-50th)	**42.4±1.07**	**144.2±0.73**
11 yo (n=4)	33.9 (10th-15th)	143.0 (10th-15th)	**49.2±1.31**	**151.3±0.69**
12 yo (n=1)	37.2 (5th-10th)	147.5 (5th-10th)	**52.9 ±1.31**	**156.7±0.55**

Bold numbers: standard weight and height referenced from CDC website.

http://www.cdc.gov/growthcharts/data/set1clinical/cj41l024.pdf.

5. Appendix

5.1 Abbreviations and Grocery

- aBMD: Areal Bone Mineral Density (g/cm^2)

  The amount of mineral matter in terms of gram per square centimeter of bones.

- ALS Acid labile subunit

  Insulin-like growth factor binding protein.

- BMAD Bone mineral apparent density

  An estimation of vertebral volumetric bone density, which is based on posteroanterior spine scan, supine lateral scan, or paired 3D scan. The paired scan is a better approximation.

- BMU Basic multicellular units

  The temporary anatomic structures which are assembled by osteoclasts and osteoblasts during bone remodeling.

- CFU-M Macrophage colony-forming units

  Osteoclast precursors that differentiated from hematopoietic stem cells.

- DXA Dual-energy X-ray absorptiometry

  A technique of measuring bone mineral density (BMD).

- DRI Dietary Reference Intake

  A system of nutrition recommendations including recommended dietary allowance, estimated average requirement, adequate intake, and tolerable upper intake level.

- FFQ Food Frequency Questionnaire

  A checklist of foods and beverages with a frequency response section for individuals to report how often each item was consumed over a specified period of time.

- IGF-1 Insulin growth factor-1

A hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults.

- IGFBP3 IGF binding protein-3

  A group of insulin growth factor binding protein that controls the bioavailability and half-life of insulin-like growth factors, in particular IGF-1, which regulates the anabolic and growth promoting effects of growth hormone.

- HA Calcium hydroxyapatite

  A naturally occurring mineral form of calcium apatite

- LAT DXA Supine lateral Dual-energy X-ray absorptiometry scans

  Dual-energy X-ray absorptiometry scans that project X-rays laterally (from side to side), while lying supine.

- MSC Mesenchymal stem cell

  Multipotent stromal cells are precursors of a variety of cell types such as osteoblasts and chondrocytes.

- NF-κB Receptor activator of nuclear factor kappa-B

  A surface-bound molecule found on osteoblasts functions to activate osteoclasts.

- OPG Osteoprotegerin

  A member of the tumor necrosis factor receptor superfamily, which can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors into osteoclasts and also regulates the resorption of osteoclasts

- PA DXA Postero-anterior dual energy X-ray absorptiometry

  Dual-energy X-ray absorptiometry scans in which X-rays are projected from back to front, while the subjects is lying supine.

- PGE Prostaglandin E_2

  A group of hormone-like lipid compounds that are derived from fatty acids.

- PTH Parathyroid hormone

  Polypeptide which is secreted by the chief cells of the parathyroid glands.

- PUFA Polyunsaturated fatty acid

  Fatty acids that contain more than one double bond in their backbone.

- pQCT Peripheral quantitative computed tomography

  A technique that measures bone mineral density.

- RANKL Binding receptor activator of nuclear factor kappa-B (NF-κB) ligand

  A receptor is the essential for osteoclast formation, fusion, activation, and survival.

- RDA Recommended Dietary Allowance

  The average daily dietary intake level which sufficiently meets the nutrient requirements of nearly all (97-98%) healthy individuals in a group.

- SoFAS Solid fat and added sugar

  Saturated fat, trans-fat, and added sugar which contain empty calories.

- SSB Sugar sweetened beverage

  A drink with added sugar.

- TGF- β Transforming growth factor-β

  A protein that controls proliferation, cellular differentiation of many cell types.

- TRPV6 Transient receptor potential vanilloid type 6

  The major ion channel in intestinal epithelial cell membranes responsible for calcium entry.

- vBMD volumetric BMD

  Volumetric density of inorganic material in terms of gram per cubic centimeter of bones.

- YAQ Youth/Adolescent Food Frequency Questionnaire

The Youth/Adolescent Food Frequency Questionnaire is a self-administered semi quantitative questionnaire that was developed by the Harvard School of Public Health to assess the dietary intakes of children and adolescent

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